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Dated: March 20, 2007 Signature: Jeanne M. Brashear
(Jeanne M. Brashear)

Docket No.: 28967/34891.1
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of: Kari Alitalo

Application No.: 10/774,802 Confirmation No.: 9059

Filed: February 9, 2004 Group Art Unit: 1647

For: FLT4 (VEGFR-3) AS A TARGET FOR TUMOR IMAGING AND ANTI-TUMOR THERAPY Examiner: Ian D. Dang

PETITION FOR WITHDRAWAL OF IMPROPER RESTRICTION REQUIREMENT PURSUANT TO 37 C.F.R. §1.181 AND §1.144

MS Petitions
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

The applicant files this petition pursuant to 37 C.F.R. §1.181 and §1.144, to request review and withdrawal of a 13-way restriction requirement imposed by the examiner and made final, notwithstanding a traversal on the merits by the applicant.

I. Statement of Facts

1. The application included a preliminary amendment dated July 24, 2004 canceling claims 1-42 and introducing new claims 43-93, which were variously directed to the following related subject matters:

> (a) methods of inhibiting Flt4 receptor tyrosine kinase (Flt4) function in a mammalian organism with a neoplastic disease, wherein the neoplastic disease is characterized by expression of Flt4 in vascular endothelial cells, (claims 43-48 and 53-70);
>
> (b) methods for antagonizing the function of Flt4 receptor tyrosine kinase (Flt4) in an organism, wherein the organism has a neoplastic disorder characterized by blood vessels comprising endothelial cells that express Flt4 (claims 49-52)
>
> (c) methods of inhibiting proliferation/genesis of blood vessel endothelial cells in a mammalian organism having a disease characterized by expression of Flt4 tyrosine kinase (Flt4) in blood vessel endothelial cells, (claims 71-84);

(d) methods of inhibiting the growth or the metastatic spread of a tumor in a mammalian organism, wherein the mammalian organism has a tumor characterized by blood and lymphatic vessels that express Flt4, (claims 85-88); and

(e) methods of inhibiting neoplastic cell growth in a human subject, wherein the neoplastic disorder characterized by blood vessels that comprise endothelial cells expressing Flt4 receptor tyrosine kinase (Flt4), (claims 89-93).

Of the 50 total claims, 13 claims are independent (claims 1, 49, 50, 53, 54, 61, 62, 65, 70-73, 78, 81, 82, 85, 86, 89, 90-92); and they are all directed to methods of inhibiting Flt4 activity in a mammal having disease characterized by Flt4 expression in blood vessel endothelial cells. A complete list of the claims as filed in the preliminary amendment dated July 23, 2004 is attached as Exhibit A. In that preliminary amendment, applicant points out that claims previously allowed in U.S. Application Serial No. 09/169,079, now U.S. Patent No. 6,824,777, from which the present application claim priority under 35 U.S.C. § 120, are generally represented in the present application. Applicant further provided a table which indicates the relationship between the newly added claims of the instant application and the allowed claims in the priority application. See pages 17-18 of Exhibit A. The issued claims of the priority application are set forth in Exhibit B.

2. On August 18, 2006, the Examiner issued the Restriction Requirement (attached as Exhibit C) that is the subject of this petition. The Examiner alleged that pending claims 43-93 were directed to 13 distinct inventions, and required restriction and election. The Examiner further alleged that the claims were directed to five species, thus restricting the application to sixty-five (13 x 5) allegedly patentably distinct inventions. In response, applicant filed an election with traverse on October 17, 2006, a copy of which is attached as Exhibit D. Issues identified in the traversal included the following:

(a) the restriction requirement failed to provide demarcations between the groups or explain how the groups are allegedly distinct from each other;

(b) the restriction requirement failed to provide reasons for restriction;

(c) the restriction was improper, insofar as certain claims were identified by the examiner in multiple restriction groups;

(c) the restriction was improper, insofar as multiple Groups were identified by the Examiner as being classified in the same class/subclass;

(e) relatedness of the various claims to elected Group X; and,

(f) the absence of serious burden, insofar as examination and allowance has already occurred on closely related claims in the parent application.

3. In the first Office action on the merits (Exhibit E), dated November 20, 2006, the Examiner maintained the Restriction Requirement, made it final, and withdrew claims that were directed to non-elected subject matter. (See Exhibit E, page 2.) In maintaining the Restriction Requirement, the Examiner indicated that "the separate classification established for each Group demonstrates that each distinct Group has attained recognition in the art as a separate subject for inventive effort, and also a separate field of search." (See page 2 of Exhibit E).

4. Groups III, IV, V, VI, VII, VIII, XI, X, XI, XII and XIII are all identified by the Examiner as classified in class 514, subclass 12. See, Exhibit C, pages 2-3.

5. Groups I and II are identified by the Examiner as classified in class 514, subclass 2 and class 514, subclass 10, respectively. See, Exhibit C, page 2.

II. Legal Authority upon Which Instant Petition is based

37 C.F.R. §1.181 provides that petition may be taken to the Commissioner in "cases in which a statute or the rules specify that the matter is to be determined directly by or reviewed by the Commissioner." (37 C.F.R. §1.181.) According to 37 C.F.R. §1.144, "[a]fter a final requirement for restriction, the applicant, in addition to making any reply due on the remainder of the action, may petition the Commissioner to review the requirement. Petition may be deferred until after final action on or allowance of claims to the invention elected, but must be filed not later than appeal."

The Patent statute only permits restriction of independent and distinct inventions (35 U.S.C. § 121), which the Patent Office interprets to permit restriction of independent "or" distinct inventions. (MPEP 803, Part I) But such restriction is proper only if there would be a "serious burden" on the examiner in the absence of the restriction. (MPEP 803; 808.02). There is no harm whatsoever in examining multiple independent or distinct inventions in one application. (MPEP 805)

Importantly, the MPEP contains numerous cautions to the effect that restriction practice is not merely a burden-easing tool to be used by examiners when examiners see fit to do so. For example, Section 803 ("Restriction – When Proper") states, "an application may properly be restricted to one of two or more claimed inventions only if they are able to support separate patents and they are either independent or distinct." Section 803.01 states, in all capital letters, "IT STILL REMAINS IMPORTANT FROM THE STANDPOINT OF THE PUBLIC INTEREST THAT NO REQUIREMENTS BE MADE WHICH MIGHT RESULT IN THE ISSUANCE OF TWO PATENTS FOR THE SAME INVENTION." Similarly, Section 804.01 states, "This apparent nullification of

double patenting as a ground of rejection or invalidity in such cases imposes a heavy burden on the Office to guard against erroneous requirements for restrictions where the claims define essentially the same invention in different language and which, if acquiesced in, might result in the issuance of several patents for the same invention." Where inventions are related as disclosed but are not distinct as claimed, restriction is never proper. (MPEP 806; MPEP 808.02) "[I]t is imperative the requirement should never be made where related inventions as claimed are not distinct." (Id.)

In view of the foregoing, the MPEP instructs that "Examiners must provide reasons and/or examples to support conclusions . . . ." (MPEP 803, Part II) See also MPEP 808.01 ("The particular reasons relied on by the examiner for holding that the inventions as claimed are either independent or distinct should be concisely stated. A mere statement of conclusion is inadequate. The reasons upon which the conclusion is based should be given. For example, relative to a combination and a subcombination thereof, the examiner should point out the reasons why he or she considers the subcombination to have utility by itself or in other combinations, and why he or she considers that the combination as claimed does not require the particulars of the subcombination as claimed. Each relationship of claimed inventions should be similarly treated and the reasons for the conclusions of distinctness or independence set forth.") "The examiner must provide a clear and detailed record of the restriction requirement to provide a clear demarcation between restricted inventions so that it can be determined whether inventions claimed in a continuing application are consonant with the restriction requirement and therefore subject to the prohibition against double patenting rejections under 35 U.S.C. 121." (MPEP 814)

III. Requested Relief and Reasons Relief Should be Granted

This petition is filed pursuant to 37 C.F.R. §1.181 and §1.144 to request review and withdrawal of the final restriction for requirement in this case. Applicant timely traversed the restriction requirement. (See Fact paragraph 2). Upon reconsideration, the Examiner made the restriction requirement final (Fact paragraph 3).

The present case was filed with 13 independent and 50 total claims, all related in that they recite a method of inhibiting Flt4 activity in a mammal having disease characterized by Flt4 expression in blood vessel endothelial cells (Fact paragraph 1).

It is both axiomatic and required by the patent statute that claims only be restricted when they define independent and distinct inventions. The current restriction requirement is defective insofar as (1) failed to provide demarcations between the groups or explain how the groups are allegedly distinct from each other; (2) the examiner failed to provide reasons for restriction; (3)

certain claims were identified by the examiner in multiple restriction groups; (4) multiple groups were identified by the examiner as being classified in the same class/subclass; and (5) the relatedness of the various claims to elected Group X. (Fact paragraphs 2, 4 and 5).

The examiner has failed to provide a clear and detailed demarcation between restriction groups. This is self-evident from comparison of, e.g., groups III and IV, which are described in *exactly the same way* ("a method of inhibiting neoplastic cell growth in a mammalian subject"). Additionally, the examiner has indicated that claims 55 and 58-60 are in both Groups. It is unclear how claims can be distinct from themselves, especially when the alleged demarcation is not a demarcation.

It should be noted that Groups III-XI and XII-XIII are also classified in the same class/subclass as elected Group X. (See Fact paragraph 4). The only justification for maintaining the restriction requirement, when it was made final, was alleged separate classification.

There are many other examples of allegedly distinct groups which, while defined by slightly varying words, are equally difficult to separate by any lines of "clear demarcation." (See, for example, the aforementioned Groups III and IV, compared to, e.g., Group VI, which is directed to a method for treating a neoplastic disorder, or Group XII, directed to a method of inhibiting neoplastic cell growth in a human, or Group XIII, directed to a method of inhibiting neoplastic cell growth in a mammalian subject.) It will be readily apparent that a claim to treating a neoplastic disorder in a human could fall within all of these groups (humans are mammals), and other groups, and it would be impossible to properly assign such a claim to one group, to the exclusion of others.

A related, fundamental defect with the restriction is a total failure to provide reasons for restriction. Instead of explaining how any single group is distinct from any other group, as directed by the MPEP, the restriction merely contains a one sentence conclusion ("The methods of groups I-XIII can be shown to be distinct as they each have different starting materials, method steps, and/or goals.") This is precisely the conclusory treatment that is prohibited by the MPEP, as quoted above.

It appears that the actual basis for restriction was whether one claim was dependent from another or independent. However, there is no justification, in the provisions for analyzing patentable distinctness, for such a mechanical analysis.

Because the Patent Office has failed to provide demarcations between the groups or explain how the groups are allegedly distinct from each other, the restriction requirement is improper, and moreover, it is exceedingly difficult to traverse on the merits. (One cannot rebut a *prima facie*

case that has not been set forth.) Notwithstanding, applicant identifies some of the other reasons for removing the restriction requirement below.

Group X is directed to "a method of inhibiting genesis of blood vessels in a mammalian organism having a disease characterized by expression of Flt4 tyrosine kinase in blood vessels. Independent claim 81 in that group includes a step of administering to a mammalian organism (having a disease characterized by expression of Flt4 in blood vessels) a composition that comprises an inhibitor of the binding between Flt4 and an Flt4 ligand. A Markush group of five classes of inhibitors is recited in the claim.

All of the other groups have varying degrees of relatedness to Group X and its claims. For example, antagonizing or inhibiting Flt4 function is a recurrent theme in the groups. Administering an inhibitor of the binding between Flt4 and its ligands is a recurrent theme in the groups. The Markush group of inhibitors recited in claim 81 is a recurrent theme in the claims of multiple groups. Flt4 expression in blood vessels (in the organism to be treated), and more particularly in the endothelia of the blood vessels, is a recurrent theme in multiple groups. The existence of neoplastic cell growth or a neoplastic disorder generally, or a breast carcinoma specifically, is a recurrent theme in multiple groups.

In order for any restriction to be maintained, it is incumbent upon the Patent Office to identify clear lines of demarcation between the groups, notwithstanding their relatedness, and define groups that maintain such demarcation. Moreover, demarcation alone is insufficient. The Patent Office also must provide reasons for concluding that the groups, so demarked, are distinct.

Moreover, applicant requests that the restriction requirement be reconsidered because the examiner has not shown that a serious burden would be required to examine all of the claims. M.P.E.P. § 803 provides, "If the search and examination of an application can be made without serious burden, the Examiner **must** examine it on the merits, even though it includes claims to distinct or independent inventions." (*Emphasis added.)* Even a *prima facie* showing of burden (e.g., by appropriate explanation of separate classification, or separate status in the art, or a different field of search as defined in MPEP § 803.02, can be rebutted by appropriate showings or evidence by the applicant.

There would be no serious burden examining all of the claims in this application at one time. Compelling evidence of this is the fact that the prior examiner examined similar claims (and more claims) to the point of allowance in the parent application. (Fact paragraph 1). The current claims (which contain limitations relating to blood vessel expression of Flt4), are believed to be

entitled to a longer patent term than the claims which were permitted to issue in the parent case, by virtue of a later priority claim. However, these claims do not pose a serious burden of examination in one application. Moreover, the relatedness, discussed above, indicates that the search for each method claim will be similar.

Likewise, the allowed claims in the parent application contain limitations directed to a variety of different Flt4 inhibitors. No serious burden was found in examining claims that recite more than one inhibitor – the second "subgroup" (A-E) prong of the restriction requirement.

IV. Conclusion

Applicant believes that the Restriction Requirement in the instant case requires review by the Commissioner, and therefore applicant requests that this petition be granted. No fee is believed to be due with the filing of a petition pursuant to 37 C.F.R. §1.144 and 37 C.F.R. §1.181, however, should a fee be deemed necessary in connection with the filing of this petition the Commissioner is hereby authorized to deduct such a fee from Marshall, Gerstein and Borun account number 13-2855, under order number 28967/34891.1.

Dated: March 20, 2007

Respectfully submitted,

By Jeanne M. Brashear

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PATENT

Attorney Docket No. 28967/34891.1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Alitalo et al.

Serial No.: 10/774,802

Filed: Herewith

For: Flt4 (VEGFR-3) as a Target for Tumor Imaging and Anti-Tumor Therapy

Examiner (Projected): Joseph F. Murphy

Group Art Unit: 1646

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Date of Deposit: **July 23, 2004**
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Juan Quintero

PRELIMINARY AMENDMENT AND CORRECTION OF INVENTORSHIP PURSUANT TO 37 C.F.R. 1.48(b)

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Sir:

Prior to examination on the merits, please enter the following preliminary amendment.

Amendments to the specification. There are no amendments to the specification.

Amendments to the claims can be found on page **2** of this paper.

Remarks begin on page **13** of this paper.

AMENDMENTS

Amendments to the Claims:

Pursuant to 37 C.F.R. 1.121 the following is a complete listing of the claims of the present application and replaces all previous versions.

1.-42. (canceled)

43. (new) A method of inhibiting Flt4 receptor tyrosine kinase (Flt4) function in a mammalian organism with a neoplastic disease, comprising the step of administering to said mammalian organism a composition,

wherein said neoplastic disease is a breast carcinoma characterized by expression of Flt4 in vascular endothelial cells,

wherein said composition comprises an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in blood vascular endothelial cells of said organism, and

wherein said composition is administered in an amount effective to inhibit Flt4-mediated proliferation of said vascular endothelial cells, thereby inhibiting Flt4-mediated proliferation of said vascular endothelial cells.

44. (new) A method according to claim 43 wherein said organism is human.

45. (new) A method according to claim 43 wherein said inhibitor comprises a polypeptide selected from the group consisting of:

(a) an anti-Flt4 antibody or a polypeptide comprising an antigen binding fragment thereof;

(b) an anti-VEGF-C antibody or a polypeptide comprising an antigen binding fragment thereof;

(c) an anti-VEGF-D antibody or a polypeptide comprising an antigen binding fragment thereof;

(d) a soluble polypeptide comprising a fragment of Flt4, wherein the polypeptide and the fragment are capable of binding to human VEGF-C (SEQ ID NO: 21); and

(e) a polypeptide comprising an Flt4 binding fragment of human prepro-VEGF-C (SEQ ID NO: 21) or human prepro-VEGF-D (SEQ ID NO: 22) conjugated to an antineoplastic agent.

46. (new) A method according to claim 43, wherein the inhibitor comprises a bispecific antibody, or fragment thereof, wherein said antibody or fragment specifically binds Flt4 and specifically binds a blood vascular endothelial marker antigen.

47. (new) A method according to claim 46 wherein said blood vascular endothelial marker antigen is selected from the group consisting of PAL-E, VEGFR-1, and VEGFR-2.

48. (new) A method according to claim 46 wherein said inhibitor further comprises an anti-neoplastic agent conjugated to said bispecific antibody.

49. (new) A method for antagonizing the function of Flt4 receptor tyrosine kinase (Flt4) in an organism, comprising a step of administering to the organism a composition comprising a polypeptide and a pharmaceutically acceptable carrier, wherein the polypeptide is selected from the group consisting of:

(a) an anti-Flt4 antibody or a polypeptide comprising an antigen binding fragment thereof;

(b) an anti-VEGF-C antibody or a polypeptide comprising an antigen binding fragment thereof;

(c) an anti-VEGF-D antibody or a polypeptide comprising an antigen binding fragment thereof;

(d) a soluble polypeptide comprising a fragment of Flt4, wherein the polypeptide and the fragment are capable of binding to human VEGF-C (SEQ ID NO: 21); and

(e) a polypeptide comprising an Flt4 binding fragment of human prepro-VEGF-C (SEQ ID NO: 21) or human prepro-VEGF-D (SEQ ID NO: 22) conjugated to an antineoplastic agent; and

wherein the organism has a neoplastic disorder characterized by blood vessels comprising endothelial cells that express Flt4.

50. (new) A method for antagonizing the function of Flt4 receptor tyrosine kinase (Flt4) in an organism, comprising a step of administration to the organism a composition comprising a bispecific antibody, or fragment thereof,

wherein said antibody or fragment specifically binds Flt4 and specifically binds a blood vascular endothelial marker antigen; and

wherein said organism has a neoplastic disorder characterized by blood vessels comprising endothelial cells that express Flt4.

51. (new) A method according to claim 49 or 50, wherein the organism is human.

52. (new) A method according to claim 50, wherein said composition further comprises an anti-neoplastic agent conjugated to said antibody or antibody fragment.

53. (new) A method of inhibiting neoplastic cell growth in a mammalian subject, comprising steps of:

(a) screening a mammalian subject to identify a neoplastic disorder characterized by blood vessels that comprise endothelial cells that express Flt4; and

(b) administering a composition to a mammalian subject identified according to step (a) as having a neoplastic disorder characterized by cells expressing Flt4, said composition comprising an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in cells of said subject, thereby inhibiting Flt4-mediated proliferation of said Flt4-expressing cells, wherein said inhibitor comprises a polypeptide selected from the group consisting of:

(i) an anti-Flt4 antibody or a polypeptide comprising an antigen binding fragment thereof;

(ii) an anti-VEGF-C antibody or a polypeptide comprising an antigen binding fragment thereof;

(iii) an anti-VEGF-D antibody or a polypeptide comprising an antigen binding fragment thereof; and

(iv) a soluble polypeptide comprising a fragment of Flt4, wherein the polypeptide and the fragment are capable of binding to human VEGF-C (SEQ ID NO: 21);

(v) a polypeptide comprising an Flt4 binding fragment of human prepro-VEGF-C (SEQ ID NO: 21) or human prepro-VEGF-D (SEQ ID NO: 22) conjugated to an antineoplastic agent.

54. (new) A method of inhibiting neoplastic cell growth in a mammalian subject, comprising steps of:

(a) screening a mammalian subject to identify a neoplastic disorder characterized by blood vessels that comprise endothelial cells that express Flt4; and

(b) administering a composition to a mammalian subject identified according to step (a) as having a neoplastic disorder characterized by cells expressing Flt4, said composition comprising an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in cells of said subject, thereby inhibiting Flt4-mediated proliferation of said Flt4-expressing cells,

wherein said inhibitor comprises a bispecific antibody, or fragment thereof, wherein said antibody or fragment specifically binds Flt4 and specifically binds a blood vascular endothelial marker antigen.

55. (new) A method according to claims 53 or 54, wherein the mammalian subject is human.

56. (new) A method according to claim 54 wherein said blood vascular endothelial marker antigen is selected from the group consisting of PAL-E, VEGFR-1, and VEGFR-2.

57. (new) A method according to claim 54 wherein said inhibitor further comprises an anti-neoplastic agent conjugated to said bispecific antibody.

58. (new) A method according to claims 53 or 54 wherein the screening step comprises:

(a) contacting tissue from the mammalian subject with a composition comprising an antibody or antibody fragment that specifically binds Flt4;

(b) detecting said antibody or antibody fragment bound to cells in said tissue; and

(c) screening for a neoplastic disorder characterized by blod vessels that comprise endothelial cells that express Flt4 from the quantity or distribution of said antibody bound to cells in said tissue.

59. (new) A method according to claim 58 wherein in said screening step, the detection of said antibody or antibody fragment bound to blood vessel endothelial cells is correlated with the presence of a neoplastic disease.

60. (new) The method according to claim 59 wherein said tissue comprises mammary tissue.

61. (new) A method of treating a mammal having breast cancer characterized by blood vessel endothelial cells that express Flt4 tyrosine kinase (Flt4), comprising a step of administering to said mammal a composition, said composition comprising an inhibitor of binding between Flt4 ligand protein and Flt4 expressed in cells of said organism, thereby inhibiting Flt4 function, wherein the inhibitor comprises a member selected from the group consisting of:

(a) an anti-Flt4 antibody or a polypeptide comprising an antigen binding fragment thereof;

(b) an anti-VEGF-C antibody or a polypeptide comprising an antigen binding fragment thereof;

(c) an anti-VEGF-D antibody or a polypeptide comprising an antigen binding fragment thereof;

(d) a soluble polypeptide comprising a fragment of Flt4, wherein the polypeptide and the fragment are capable of binding to human VEGF-C (SEQ ID NO: 21); and

(e) a polypeptide comprising an Flt4 binding fragment of human prepro-VEGF-C (SEQ ID NO: 21) or human prepro-VEGF-D (SEQ ID NO: 22) conjugated to an antineoplastic agent.

62. (new) A method of treating a mammal having breast cancer characterized by blood vessel endothelial cells that express Flt4 tyrosine kinase (Flt4), comprising a step of administering to said mammal a composition, said composition comprising an inhibitor of

binding between Flt4 ligand protein and Flt4 expressed in cells of said organism, thereby inhibiting Flt4 function,

wherein the inhibitor comprises a bispecific antibody, or fragment thereof, wherein said antibody or fragment specifically binds Flt4 and specifically binds a blood vascular endothelial marker antigen.

63. (new) A method according to claims 61 or 62 wherein said mammal is human.

64. (new) A method according to claim 61 or 62 comprising a screening step preceding the administering step, wherein the screening step comprises screening a human to identify breast cancer characterized by blood vessel endothelial cells expressing Flt4; and

wherein the administering step comprises administering the composition to a human identified by the screening step as having breast cancer characterized by blood vessel endothelial cells expressing Flt4.

65. (new) A method for treating a neoplastic disorder in a mammalian subject, comprising the steps of:

(a) screening a mammalian subject to identify a neoplastic disorder characterized by blood vessel endothelial cells expressing Flt4 receptor tyrosine kinase (Flt4); and

(b) administering a composition to a mammalian organism identified according to step (a) as having a neoplastic disorder characterized by blood vessel endothelial cells expressing Flt4, to inhibit Flt4 mediated proliferation of said Flt4-expressing cells,

wherein the composition comprises a means for inhibiting Flt4 function in mixture with a pharmaceutically acceptable diluent, adjuvant, or carrier.

66. (new) A method according to claim 65, wherein the mammalian subject is human.

67. (new) A method according to claim 66, wherein the means for inhibiting comprises a member selected from the group consisting of:

(a) an anti-Flt4 antibody or a polypeptide comprising an antigen binding fragment thereof;

(b) an anti-VEGF-C antibody or a polypeptide comprising an antigen binding fragment thereof;

(c) an anti-VEGF-D antibody or a polypeptide comprising an antigen binding fragment thereof;

(d) a soluble polypeptide comprising a fragment of Flt4, wherein the polypeptide and the fragment are capable of binding to human VEGF-C (SEQ ID NO: 21); and

(e) a polypeptide comprising an Flt4 binding fragment of human prepro-VEGF-C (SEQ ID NO: 21) or human prepro-VEGF-D (SEQ ID NO: 22) conjugated to an antineoplastic agent.

68. (new) A method according to claim 66, wherein the means for inhibiting comprises a bispecific antibody, or fragment thereof, wherein said antibody or fragment specifically binds Flt4 and specifically binds a blood vascular endothelial marker antigen.

69. (new) A method according to claim 68 wherein said blood vascular endothelial marker antigen is selected from the group consisting of PAL-E, VEGFR-1, and VEGFR-2.

70. (new) A method according to claim 68 wherein the means for inhibiting further comprises an anti-neoplastic agent conjugated to said bispecific antibody.

71. (new) A method of inhibiting proliferation of blood vessel endothelial cells in a mammalian organism having a disease characterized by expression of Flt4 tyrosine kinase (Flt4) in blood vessel endothelial cells, comprising the step of administering to said mammalian organism a composition, said composition comprising an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in blood vessel endothelial cells of said organism, thereby inhibiting Flt4-mediated proliferation of the blood vessel endothelial cells, wherein said inhibitor comprises a polypeptide selected from the group consisting of:

(a) an anti-Flt4 antibody or a polypeptide comprising an antigen binding fragment thereof;

(b) an anti-VEGF-C antibody or a polypeptide comprising an antigen binding fragment thereof;

(c) an anti-VEGF-D antibody or a polypeptide comprising an antigen binding fragment thereof;

(d) a soluble polypeptide comprising a fragment of Flt4, wherein the polypeptide and the fragment are capable of binding to human VEGF-C (SEQ ID NO: 21); and

(e) a polypeptide comprising an Flt4 binding fragment of human prepro-VEGF-C (SEQ ID NO: 21) or human prepro-VEGF-D (SEQ ID NO: 22) conjugated to an antineoplastic agent.

72. (new) A method of inhibiting proliferation of blood vessel endothelial cells in a mammalian organism having a disease characterized by expression of Flt4 tyrosine kinase (Flt4) in blood vessel endothelial cells, comprising the step of administering to said mammalian organism a composition, said composition comprising an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in blood vessel endothelial cells of said organism, thereby inhibiting Flt4-mediated proliferation of the blood vessel endothelial cells,

wherein the inhibitor comprises a bispecific antibody, or fragment thereof, wherein said antibody or fragment specifically binds Flt4 and specifically binds a blood vascular endothelial marker antigen.

73. (new) A method of inhibiting proliferation of endothelial cells in a human organism having a disease characterized by expression of Flt4 tyrosine kinase (Flt4) in endothelial cells, comprising the step of administering to said human organism a composition, said composition comprising an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in endothelial cells of said organism, thereby inhibiting Flt4-mediated proliferation of the cells, wherein the inhibitor comprises a bispecific antibody, or fragment thereof, wherein said antibody or fragment specifically binds Flt4 and specifically binds a blood vascular endothelial marker antigen.

74. (new) A method according to claim 72 or 73, wherein said blood vascular endothelial marker antigen is selected from the group consisting of PAL-E, VEGFR-1, and VEGFR-2.

75. (new) A method according to claim 72 or 73, wherein said inhibitor further comprises an anti-neoplastic agent conjugated to said bispecific antibody.

76. (new) A method according to claim 72 or 73, wherein the disease is a tumor characterized by blood vessels with endothelial cells expressing Flt4.

77. (new) A method according to claim 66, wherein the disease is a breast carcinoma characterized by expression of Flt4 in vascular endothelial cells.

78. (new) A method of inhibiting proliferation of endothelial cells in a human organism having a breast carcinoma characterized by expression of Flt4 tyrosine kinase (Flt4) in vascular endothelial cells, comprising the step of administering to said human organism a composition, said composition comprising an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in endothelial cells of said organism, thereby inhibiting Flt4-mediated proliferation of the cells, wherein the inhibitor comprises a bispecific antibody, or fragment thereof, wherein said antibody or fragment specifically binds Flt4 and specifically binds a blood vascular endothelial marker antigen.

79. (new) A method according to claim 78 wherein said blood vascular endothelial marker antigen is selected from the group consisting of PAL-E, VEGFR-1, and VEGFR-2.

80. (new) A method according to claim 78 wherein said inhibitor further comprises an anti-neoplastic agent conjugated to said bispecific antibody.

81. (new) A method of inhibiting genesis of blood vessels in a mammalian organism having a disease characterized by expression of Flt4 tyrosine kinase (Flt4) in blood vessels, comprising the step of administering to said mammalian organism a composition, said composition comprising an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in cells of said organism, thereby inhibiting Flt4-mediated proliferation of the cells, wherein said inhibitor comprises a member selected from the group consisting of:

(a) an anti-Flt4 antibody or a polypeptide comprising an antigen binding fragment thereof;

(b) an anti-VEGF-C antibody or a polypeptide comprising an antigen binding fragment thereof;

(c) an anti-VEGF-D antibody or a polypeptide comprising an antigen binding fragment thereof;

(d) a soluble polypeptide comprising a fragment of Flt4, wherein the polypeptide and the fragment are capable of binding to human VEGF-C (SEQ ID NO: 21); and

(e) a polypeptide comprising an Flt4 binding fragment of human prepro-VEGF-C (SEQ ID NO: 21) or human prepro-VEGF-D (SEQ ID NO: 22) conjugated to an antineoplastic agent.

82. (new) A method of inhibiting genesis of blood vessels in a mammalian organism having a disease characterized by expression of Flt4 tyrosine kinase (Flt4) in blood vessels, comprising the step of administering to said mammalian organism a composition, said composition comprising an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in cells of said organism, thereby inhibiting Flt4-mediated proliferation of the cells,

wherein the inhibitor comprises a bispecific antibody, or fragment thereof, wherein said antibody or fragment specifically binds Flt4 and specifically binds a blood vascular endothelial marker antigen.

83. (new) A method according to claim 81 or 82 wherein said organism is human.

84. (new) A method according to claim 83, wherein the human has a tumor characterized by blood vessels that express Flt4.

85. (new) A method of inhibiting the growth or the metastatic spread of a tumor in a mammalian organism, comprising steps of administering to a mammalian organism a composition that comprises an inhibitor of the binding of an Flt4 ligand protein to Flt4 receptor tyrosine kinase (Flt4) expressed in cells of said organism, wherein the mammalian organism has a tumor characterized by blood and lymphatic vessels that express Flt4, and wherein the composition inhibits proliferation of the blood and lymphatic vessels, thereby inhibiting growth or metastatic spread of the tumor, wherein the inhibitor comprises a member selected from the group consisting of:

(a) an anti-Flt4 antibody or a polypeptide comprising an antigen binding fragment thereof;

(b) an anti-VEGF-C antibody or a polypeptide comprising an antigen binding fragment thereof;

(c) an anti-VEGF-D antibody or a polypeptide comprising an antigen binding fragment thereof;

(d) a soluble polypeptide comprising a fragment of Flt4, wherein the polypeptide and the fragment are capable of binding to human VEGF-C (SEQ ID NO: 21); and

(e) a polypeptide comprising an Flt4 binding fragment of human prepro-VEGF-C (SEQ ID NO: 21) or human prepro-VEGF-D (SEQ ID NO: 22) conjugated to an antineoplastic agent.

86. (new) A method of inhibiting the growth or the metastatic spread of a tumor in a mammalian organism, comprising steps of administering to a mammalian organism a composition that comprises an inhibitor of the binding of an Flt4 ligand protein to Flt4 receptor tyrosine kinase (Flt4) expressed in cells of said organism, wherein the mammalian organism has a tumor characterized by blood and lymphatic vessels that express Flt4, and wherein the composition inhibits proliferation of the blood and lymphatic vessels, thereby inhibiting growth or metastatic spread of the tumor,

wherein the inhibitor comprises a bispecific antibody, or fragment thereof, wherein said antibody or fragment specifically binds Flt4 and specifically binds a blood vascular endothelial marker antigen.

87. (new) A method according to claim 85 or 86, comprising a diagnosing step prior to the administering step, said diagnosing step comprising identifying a patient having a tumor characterized by blood vessels that express Flt4.

88. (new) A method according to claim 85 or 86, comprising a diagnosing step prior to the administering step, said diagnosing step comprising identifying a patient having lymph node metastasis of a tumor, wherein the lymph node comprises cells expressing Flt4.

89. (new) A method of inhibiting neoplastic cell growth in a human subject, comprising steps of:

(a) screening a human subject to identify a neoplastic disorder characterized by blood vessels that comprise endothelial cells expressing Flt4 receptor tyrosine kinase (Flt4); and

(b) administering a composition to a human subject identified according to step (a) as having a neoplastic disorder characterized by blood vessels that comprise endothelial cells expressing Flt4, said composition comprising an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in cells of said subject, thereby inhibiting Flt4-mediated proliferation of said Flt4-expressing cells, wherein the inhibitor comprises a member selected from the group consisting of:

(i) an anti-Flt4 antibody or a polypeptide comprising an antigen binding fragment thereof;

(ii) an anti-VEGF-C antibody or a polypeptide comprising an antigen binding fragment thereof;

(iii) an anti-VEGF-D antibody or a polypeptide comprising an antigen binding fragment thereof;

(iv) a soluble polypeptide comprising a fragment of Flt4, wherein the polypeptide and the fragment are capable of binding to human VEGF-C (SEQ ID NO: 21); and

(v) a polypeptide comprising an Flt4 binding fragment of human prepro-VEGF-C (SEQ ID NO: 21) or human prepro-VEGF-D (SEQ ID NO: 22) conjugated to an antineoplastic agent.

90. (new) A method of inhibiting neoplastic cell growth in a human subject, comprising steps of:

(a) screening a human subject to identify a neoplastic disorder characterized by blood vessels that comprise endothelial cells expressing Flt4 receptor tyrosine kinase (Flt4); and

(b) administering a composition to a human subject identified according to step (a) as having a neoplastic disorder characterized by blood vessels that comprise endothelial cells expressing Flt4, said composition comprising an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in cells of said subject, thereby inhibiting Flt4-mediated proliferation of said Flt4-expressing cells,

wherein the inhibitor comprises a bispecific antibody, or fragment thereof, wherein said antibody or fragment specifically binds Flt4 and specifically binds a blood vascular endothelial marker antigen.

91. (new) A method of inhibiting neoplastic cell growth in a mammalian subject, comprising steps of:

(a) screening a mammalian subject to identify a neoplastic disorder characterized by blood vessel endothelial cells expressing Flt4 receptor tyrosine kinase (Flt4), wherein the screening comprises

(i) contacting tissue from the mammalian subject with a composition comprising an antibody or antibody fragment that specifically binds Flt4;

(ii) detecting said antibody or antibody fragment bound to cells in said tissue; and

(iii) screening for a neoplastic disorder from the quantity or distribution of said antibody or antibody fragment bound to cells in said tissue, wherein the detection of said antibody or antibody fragment bound to blood vessel endothelial cells is correlated with the presence of a neoplastic disease; and

(b) administering a composition to a mammalian subject identified according to step (a) as having a neoplastic disorder characterized by blood vessel endothelial cells expressing Flt4, said composition comprising an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in cells of said subject, thereby inhibiting Flt4-mediated proliferation of said Flt4-expressing cells, wherein the inhibitor comprises a member selected from the group consisting of:

(i) an anti-Flt4 antibody or a polypeptide comprising an antigen binding fragment thereof;

(ii) an anti-VEGF-C antibody or a polypeptide comprising an antigen binding fragment thereof;

(iii) an anti-VEGF-D antibody or a polypeptide comprising an antigen binding fragment thereof;

(iv) a soluble polypeptide comprising a fragment of Flt4, wherein the polypeptide and the fragment are capable of binding to human VEGF-C (SEQ ID NO: 21); and

(v) a polypeptide comprising an Flt4 binding fragment of human prepro-VEGF-C (SEQ ID NO: 21) or human prepro-VEGF-D (SEQ ID NO: 22) conjugated to an antineoplastic agent.

92. (new) A method of inhibiting neoplastic cell growth in a mammalian subject, comprising steps of:

(a) screening a mammalian subject to identify a neoplastic disorder characterized by blood vessel endothelial cells expressing Flt4 receptor tyrosine kinase (Flt4), wherein the screening comprises

(i) contacting tissue from the mammalian subject with a composition comprising an antibody or antibody fragment that specifically binds Flt4;

(ii) detecting said antibody or antibody fragment bound to cells in said tissue; and

(iii) screening for a neoplastic disorder from the quantity or distribution of said antibody or antibody fragment bound to cells in said tissue, wherein the detection of said antibody or antibody fragment bound to blood vessel endothelial cells is correlated with the presence of a neoplastic disease; and

(b) administering a composition to a mammalian subject identified according to step (a) as having a neoplastic disorder characterized by blood vessel endothelial cells expressing Flt4, said composition comprising an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in cells of said subject, thereby inhibiting Flt4-mediated proliferation of said Flt4-expressing cells,

wherein the inhibitor comprises a bispecific antibody, or fragment thereof, wherein said antibody or fragment specifically binds Flt4 and specifically binds a blood vascular endothelial marker antigen.

93. (new) A method according to claim 91 or 92, wherein said tissue comprises mammary tissue.

REMARKS

Claims 1-42 have been canceled. Applicants point out that claims previously allowed in related application U.S. Serial No. 09/169,079 (see, for example, the Office action mailed on April 8, 2003, wherein claims 1-3, 5-14, 43-57, 59-79, 89-93, 95-112, and 123-131 were deemed allowable) are generally represented in the instant application. For the Examiner's convenience, the following table indicates the relationship between the newly added claims of the instant application and the claims in the aforementioned related application U.S. Serial no. 09/169,079.

Claim No. in instant application	Claim No. in U.S. Serial No. 09/169,079
43	10
44	10
45	10, 123
46	10, 123
47	14
48	14
49	47, 123
50	47, 123
51	47, 123
52	47, 123
53	62, 123
54	62, 123
55	62
56	63
57	63
58	70
59	70
60	71
61	76, 123
62	76, 123
63	76
64	76
65	87
66	87
67	87, 123
68	87, 123
69	87, 63
70	87, 63
71	92, 123
72	92, 123
73	100
74	101

75	102
76	105
77	106
78	108
79	109
80	110
81	112, 113
82	112, 113
83	113
84	115
85	120
86	120
87	121
88	122
89	123
90	123
91	124
92	124
93	125

The Applicant does not intend by these claim amendments or any other amendments to abandon the subject matter of any claim as originally filed or as later presented. The Applicant reserves the right to pursue such subject matter in subsequent applications, such as continuations, CIP's, and divisionals. The amendment includes no new matter.

REQUEST TO CORRECT INVENTORSHIP

As a result of the claim amendments made herein, the Applicant hereby acknowledges that the following named inventor's inventions are no longer being claimed in the instant application: Arja Kaipainen, Reija Valtola, and Lotta Jussila. Accordingly, the Applicant respectfully requests the deletion of the aforesaid names of inventors pursuant to 37 C.F.R. 1.48(b). The processing fee set forth in 1.17(i) is enclosed herewith.

Respectfully submitted,

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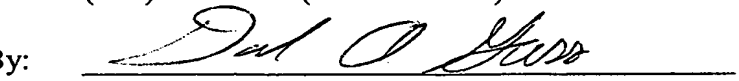
Dated: **July 23, 2004** By: 

David A. Gass
Registration No. 38,153

-continued

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            260                 265                 270

Cys Lys Thr Pro Cys Pro Lys Asp Leu Ile Gln His Pro Lys Asn Cys
        275                 280                 285

Ser Cys Phe Glu Cys Lys Glu Ser Leu Glu Thr Cys Cys Gln Lys His
    290                 295                 300

Lys Leu Phe His Pro Asp Thr Cys Ser Cys Glu Asp Arg Cys Pro Phe
305                 310                 315                 320

His Thr Arg Pro Cys Ala Ser Gly Lys Thr Ala Cys Ala Lys His Cys
                325                 330                 335

Arg Phe Pro Lys Glu Lys Arg Ala Ala Gln Gly Pro His Ser Arg Lys
            340                 345                 350

Asn Pro
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What is claimed is:

1. A method of inhibiting Flt4 receptor tyrosine kinase (Flt4) function in a mammalian organism, comprising the step of administering to said mammalian organism a composition, said composition comprising an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in cells of said organism, thereby inhibiting Flt4 function, wherein said inhibitor comprises a polypeptide selected from the group consisting of:

(a) a polypeptide comprising an antigen binding fragment of an anti Flt4 antibody; and

(b) a polypeptide comprising a soluble Flt4 fragment, wherein said fragment and said polypeptide are capable of binding to an Flt4 ligand.

2. A method according to claim 1 wherein said cells comprise endothelial cells.

3. A method according to claim 2 wherein said organism is human.

4. A method according to claim 3, wherein said composition further comprises a pharmaceutically acceptable diluent, adjuvant, or carrier medium.

5. A method according to claim 3 wherein said inhibitor comprises an anti Flt4 antibody or fragment thereof that binds to Flt4.

6. A method according to claim 5 wherein said inhibitor further comprises an anti-neoplastic agent conjugated to said antibody or antibody fragment.

7. A method according to claim 3 wherein said organism has a neoplastic disease characterized by expression of Flt4 tyrosine kinase (Flt4) in vascular endothelial cells,

wherein said composition comprises an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in vascular endothelial cells of said organism, and

wherein said composition is administered in an amount effective to inhibit Flt4-mediated proliferation of said vascular endothelial cells, thereby inhibiting Flt4-mediated proliferation of said vascular endothelial cells.

8. A method according to claim 7 wherein said neoplastic disease is selected from the group consisting of carcinomas, squamous cell carcinomas, lymphomas, melanomas, and sarcomas.

9. A method according to claim 7 wherein said inhibitor comprises a polypeptide comprising an antigen-binding fragment of an anti-Flt4 antibody.

10. A method for antagonizing the function of Flt4 receptor tyrosine kinase (Flt4) in an organism that expresses Flt4, comprising a step of administering to the organism a composition comprising a Flt4 antibody or Flt4 binding fragment thereof in a pharmaceutically acceptable carrier.

11. A method according to claim 10, wherein the organism is human.

12. A method according to claims 11 wherein said composition further comprises an anti-neoplastic agent conjugated to said antibody or antibody fragment.

13. A method according to claim 11, wherein the organism has a neoplastic disorder characterized by lymphatic vessels comprising lymphatic endothelia that express Flt4.

14. A method for antagonizing the function of Flt4 receptor tyrosine kinase (Flt4) in an organism that expresses Flt4, comprising a step of administering to the organism a composition comprising a soluble fragment of Flt4 in a pharmaceutically acceptable carrier, wherein the fragment binds to a Flt4 ligand.

15. A method according to claim 14, wherein the organism is human.

16. A method according to claim 15, wherein the organism has a neoplastic disorder characterized by lymphatic vessels comprising lymphatic endothelia that express Flt4.

17. A method of inhibiting neoplastic cell growth in a mammalian subject, comprising steps of:

(a) screening a mammalian subject to identify a neoplastic disorder characterized by cells expressing Flt4 receptor tyrosine kinase (Flt4); and

(b) administering a composition to a mammalian subject identified according to step (a) as having a neoplastic disorder characterized by cells expressing Flt4, said composition comprising an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in cells of said subject, thereby inhibiting Flt4-mediated proliferation of said Flt4-expressing cells, wherein said inhibitor comprises a polypeptide selected from the group consisting of:

(i) a polypeptide comprising an antigen binding fragment of an anti Flt4 antibody; and

(ii) a polypeptide comprising a soluble Flt4 fragment, wherein said fragment and said polypeptide are capable of binding to an Flt4 ligand.

18. A method according to claim 17, wherein the mammalian subject is human.

19. A method according to claim 18, wherein step (a) comprises screening for a neoplastic disorder characterized by undesirable lymphatic vessels comprising lymphatic endothelia that express Flt4.

20. A method according to claim 18, wherein step (a) comprises screening for a neoplastic disorder characterized by neoplastic cells that express Flt4.

21. A method according to claim 20, wherein the neoplastic cells comprise lymphoma cells that express Flt4.

22. A method according to claim 18 wherein said composition further comprises a pharmaceutically acceptable diluent, adjuvant, or carrier medium.

23. A method according to claim 18 wherein said inhibitor comprises an anti-Flt4 antibody or fragment thereof.

24. A method according to claim 18, wherein the inhibitor comprises an extracellular domain fragment of human Fit4.

25. A method according to claim 18, wherein the screening step comprises:

(a) contacting tissue from the human subject with a composition comprising an Flt4 binding compound; and

(b) screening for a neoplastic disorder characterized by cells expressing Flt4 by detecting said Flt4 binding compound bound to said tissue.

26. A method according to claim 25 wherein said Flt4 binding compound is an antibody that specifically binds Flt4 or an antigen-binding fragment thereof.

27. A method according to claim 26, wherein said antibody or fragment further comprises a detectable label covalently bound thereto.

28. A method according to claim 18, wherein the screening step comprises:

(a) administering a composition to the human subject, said composition comprising an antibody that specifically binds Flt4 or an antigen-binding fragment thereof; and

(b) screening for a neoplastic disorder characterized by cells expressing Flt4 by detecting said antibody or said fragment bound to cells in said human subject, thereby detecting Flt4 expressed on the surface of cells in said human subject.

29. A method according to claim 28, wherein said antibody or antibody fragment further comprises a detectable label.

30. A method according to claim 17, wherein the screening step comprises:

(a) contacting tissue from the mammalian subject with a composition comprising an antibody or antibody fragment that specifically binds Flt4;

(b) detecting said antibody or antibody fragment bound to cells in said tissue; and

(c) screening for a neoplastic disorder from the quantity or distribution of said antibody bound to cells in said tissue.

31. A method according to claim 30, wherein in said screening step, the screening comprises measuring the quantity or distribution of said antibody bound to lymphatic vessels.

32. A method of treating a mammal having breast cancer characterized by endothelial cells that express Flt4 tyrosine kinase (Flt4), comprising a step of administering to said mammal a composition, said composition comprising an inhibitor of binding between Flt4 ligand protein and Flt4 expressed in cells of said organism, thereby inhibiting Flt4 function, wherein the inhibitor comprises a member selected from the group consisting of:

(a) an anti-Flt4 antibody or a polypeptide comprising an antigen binding fragment thereof;

(b) an anti-VEGF-C antibody or a polypeptide comprising an antigen binding fragment thereof;

(c) an anti-VEGF-D antibody or a polypeptide comprising an antigen binding fragment thereof; and

(d) a soluble polypeptide comprising a fragment of Flt4, wherein the polypeptide and the fragment are capable of binding to human VEGF-C (SEQ ID NO: 21).

33. A method according to claim 32, wherein the mammal is human.

34. A method according to claim 33, comprising a screening step preceding the administering step,

wherein the screening step comprises screening a human to identify breast cancer characterized by endothelial cells expressing Flt4; and

wherein the administering step comprises administering the composition to a human identified by the screening step as having breast cancer characterized by endothelial cells expressing Flt4.

35. A method according to any one of claims 33–34 wherein the inhibitor comprises a member selected from the group consisting of:

(a) an anti-Flt4 antibody or a polypeptide comprising an antigen binding fragment thereof; and

(b) a soluble polypeptide comprising a fragment of Flt4, wherein the polypeptide and the fragment are capable of binding to human VEGF-C (SEQ ID NO: 21).

36. A method according to claim 32, wherein the inhibitor further comprises an anti-neoplastic agent conjugated to the antibody or polypeptide.

37. A method according to claim 32, wherein the composition further comprises a pharmaceutically acceptable diluent, adjuvant, or carrier.

38. A method of inhibiting proliferation of cells in a mammalian organism having a disease characterized by expression of Flt4 tyrosine kinase (Flt4) in cells, comprising the step of administering to said mammalian organism a composition, said composition comprising an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in cells of said organism, thereby inhibiting Flt4-mediated proliferation of the cells, wherein said inhibitor comprises a polypeptide selected from the group consisting of:

(a) a polypeptide comprising an antigen binding fragment of an anti Flt4 antibody; and

(b) a polypeptide comprising a soluble Flt4 fragment, wherein said fragment and said polypeptide are capable of binding to an Flt4 ligand.

39. A method according to claim 38 wherein said cells comprise endothelial cells.

40. A method according to claim 39, wherein said cells comprise lymphatic endothelial cells, and said composition inhibits lymphatic vascularization.

41. A method according to claim 39 wherein said organism is human.

42. A method according to claim 38 wherein said composition further comprises a pharmaceutically acceptable diluent, adjuvant, or carrier medium.

43. A method according to claim 41 wherein said inhibitor comprises an anti Flt4 antibody or fragment thereof that binds to Flt4.

44. A method according to claim 43 wherein said inhibitor further comprises an anti-neoplastic agent conjugated to said antibody or antibody fragment.

45. A method according to claim 41, wherein the inhibitor comprises an anti-Flt4 antibody.

46. A method according to claim 41 wherein the inhibitor comprises a polypeptide comprising an antigen-binding fragment of an anti-Flt4 antibody.

47. A method according to claim 41 wherein said neoplastic disease is selected from the group consisting of carcinomas, squamous cell carcinomas, lymphomas, melanomas, and sarcomas.

48. A method according to claim 42 wherein the inhibitor comprises a polypeptide comprising an antigen-binding fragment of an anti-Flt4 antibody.

49. A method according to claim 41, wherein the disease is a cancer characterized by metastatic lymph nodes.

50. A method of inhibiting genesis of lymphatic vessels in a mammalian organism having a disease characterized by expression of Flt4 tyrosine kinase (Flt4) in lymphatic vessels, comprising the step of administering to said mammalian organism a composition, said composition comprising an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in cells of said organism, thereby inhibiting Flt4-mediated proliferation of the cells, wherein said inhibitor comprises a member selected from the group consisting of:

(a) an anti-Flt4 antibody;

(b) a polypeptide comprising an antigen binding fragment of an anti Flt4 antibody; and

(c) a polypeptide comprising a soluble Flt4 fragment, wherein said fragment and said polypeptide are capable of binding to an Flt4 ligand.

51. A method according to claim 50 wherein said organism is human.

52. A method according to claim 51, wherein the human has a cancer characterized by lymph node metastases.

53. A method of treating a human having breast cancer characterized by endothelial cells that express Flt4 tyrosine kinase (Flt4), comprising a step of administering to said human a composition, said composition comprising an inhibitor of binding between Flt4 ligand protein and Flt4 expressed in cells of said human, thereby inhibiting Flt4 function,

wherein the inhibitor comprises a polypeptide comprising an Flt4 binding fragment of human prepro-VEGF-C (SEQ ID NO: 21) or human prepro-VEGF-D (SEQ ID NO: 22) conjugated to an antineoplastic agent.

54. A method according to claim 53, comprising a screening step preceding the administering step,

wherein the screening step comprises screening a human to identify breast cancer characterized by endothelial cells expressing Flt4; and

wherein the administering step comprises administering the composition to a human identified by the screening step as having breast cancer characterized by endothelial cells expressing Flt4.

* * * * *

Office Action Summary	Application No. 10/774,802	Applicant(s) ALITALO, KARI
	Examiner Ian Dang	Art Unit 1647

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 1 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) 43-93 is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☐ Claim(s) _____ is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☒ Claim(s) 43-93 are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

Election/Restrictions

Restriction to one of the following inventions is required under 35 U.S.C. 121:

I. Claims 43-48, drawn to a method of inhibiting Flt4 receptor tyrosine kinase function in a mammalian organism with a neoplastic disease comprising administering an inhibitor of the binding of a Flt4 ligand protein to Flt4 expressed in blood vascular endothelial cells, classified in class 514, subclass 2.

II. Claims 49-52, drawn to a method for antagonizing the function of Flt4 receptor tyrosine kinase in an organism comprising administering a polypeptide and a pharmaceutically acceptable carrier, classified in class 514, subclass 10.

III. Claim 53, 55, and 58-60 (in part) drawn to a method of inhibiting neoplastic cell growth in a mammalian subject, classified in class 514, subclass 12.

IV. Claims 54-55 (in part), 56-57, 58-60 (in part) drawn to a method of inhibiting neoplastic cell growth in a mammalian subject, classified in class 514, subclass 12.

V. Claims 61-64, drawn to a method of treating a mammal having breast cancer comprising administering an inhibitor of binding between Flt4 ligand protein and Flt4 expressed in cells, classified in class 514, subclass 12.

VI. Claims 65-70 and 77, drawn to a method for treating a neoplastic disorder comprising of screening a mammalian subject to identify a neoplastic disorder and administering a composition to a mammalian organism, classified in class 514, subclass 12.

VII. Claims 71-72, and 74-76, drawn to a method of inhibiting proliferation of blood vessels endothelial cells in a mammalian organism, classified in class 514, subclass 12.

VIII. Claims 73-76 drawn to a method of inhibiting proliferation of endothelial cells in a human organism having a disease characterized by expression of Flt4 tyrosine kinase (Flt4) in endothelial cells, classified in class 514, subclass 12.

XIX. Claims 78-80, drawn to a method of inhibiting proliferation of endothelial cells in a human organism having a breast carcinoma characterized by expression of Flt4 tyrosine kinase (Flt4) in vascular endothelial cells, classified in class 514, subclass 12.

X. Claims 81-84, drawn to a method of inhibiting genesis of blood vessels in a mammalian organism having a disease characterized by expression of Flt4 tyrosine kinase (Flt4) in blood vessels, classified in class 514, subclass 12.

XI. Claims 85-88, drawn to a method of inhibiting the growth or the metastatic spread of a tumor in a mammalian organism, classified in class 514, subclass 12.

XII. Claims 89-90, drawn to a method of inhibiting neoplastic cell growth in a human subject, classified in class 514, subclass 12.

XIII. Claims 91-93, drawn to a method of inhibiting neoplastic cell growth in mammalian subject, classified in class 514, subclass 12.

If one of Groups I-XIII is elected, then election is also required to one of inventions (A)-(E). The inventions of subgroups (A)-(E) represent the elected invention wherein the inhibitor is one of the following polypeptide:

(A) an anti-Flt4 antibody or a polypeptide comprising an antigen binding fragment

(B) an anti-VEGF-C antibody or a polypeptide comprising an antigen binding fragment

(C) an anti-VEGF-D antibody or a polypeptide comprising an antigen binding fragment

(D) a soluble polypeptide comprising a fragment of Flt4, wherein the polypeptide and the fragment are capable of binding to human VEGF-C

(E) a polypeptide comprising an Flt4 binding fragment of human prepro-VEGF-C or human prepro-VEGF-D conjugated to an antineoplastic agent

The inventions are independent or distinct, each from the other because:

The methods of groups I-XIII can be shown to be distinct as they each have different starting materials, methods steps, and/or goals. Each of the methods can be shown to be distinct from each of the products in that the product is either not used by the method or can be used in multiple methods.

Because these inventions are independent or distinct for the reasons given above and have acquired a separate status in the art because of the art and their recognized divergent subject matter, the inventions require a different field of search, (see MPEP § 808.02), and need for non-coextensive non-patent literature search, restriction for examination purposes as indicated is proper. Further, note that the prohibition against double patenting rejections of 35 U.S.C. 121 does not apply where the restriction requirement is withdrawn by the examiner before the patent issues. See MPEP 804.01

Applicant is advised that the reply to this requirement to be complete must include an election of the invention to be examined even though the requirement be traversed (37 CFR 1.143).

Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be amended in compliance with 37 CFR 1.48(b) if one or more of the

currently named inventors is no longer an inventor of at least one claim remaining in the application. Any amendment of inventorship must be accompanied by a request under 37 CFR 1.48(b) and by the fee required under 37 CFR 1.17(i).

Information

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Ian Dang whose telephone number is (571) 272-5014. The examiner can normally be reached on Monday-Friday 9am to 5pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Brenda Brumback can be reached on (571) 272-0961. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

Ian Dang
Patent Examiner
Art Unit 1647
August 14, 2006

Brenda Brumback
BRENDA BRUMBACK
SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 1600

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted by facsimile to the Patent and Trademark Office, facsimile no. (571) 273-8300, on the date shown below.

Dated: October 17, 2006 Signature: Jeanne M. Brashear (Jeanne M. Brashear)

Docket No.: 28967/34891.1
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Kari Alitalo et al.

Application No.: 10/774,802 Confirmation No.: 9059

Filed: February 9, 2004 Art Unit: 1646

For: FLT4 (VEGFR-3) AS A TARGET FOR TUMOR IMAGING AND ANTI-TUMOR THERAPY Examiner: Dang, Ian D.

RESPONSE TO RESTRICTION REQUIREMENT

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

This paper is in response to the restriction requirement set forth in the Office Action mailed August 18, 2006. It is timely filed with a petition and fee for one-month extension of time.

This paper contains no amendments.

Remarks begin at page 2.

REMARKS

Restriction Requirement

In the restriction requirement, the examiner required the election of one of the sixty-five (13 x 5) allegedly patentably distinct inventions:

Group I: Claims 43-48, drawn to a method of inhibiting Flt4 receptor tyrosine kinase function in a mammalian organism with a neoplastic disease comprising administering an inhibitor of the binding of a Flt4 ligand protein to Flt4 expressed in blood vascular endothelial cells;

Group II: Claims 49-52, drawn to a method for antagonizing the function of Flt4 receptor tyrosine kinase in an organism comprising administering a polypeptide and a pharmaceutically acceptable carrier;

Group III: Claims 53, 55, and 58-60 (in part), drawn to a method of inhibiting neoplastic cell growth in a mammalian subject;

Group IV: Claims 54-55 (in part), 56-57, 58-60 (in part), drawn to a method of inhibiting neoplastic cell growth in a mammalian subject;

Group V: Claims 61-64, drawn to a method for treating a mammal having breast cancer comprising administering an inhibitor of the binding of a Flt4 ligand protein to Flt4 expressed in cells;

Group IV: Claims 65-70 and 77, drawn to a method for treating a neoplastic disorder comprising screening a mammalian subject to identify a neoplastic disorder and comprising a composition to a mammalian organism;

Group VII: Claims 71-72 and 74-76, drawn to a method of inhibiting proliferation of blood vessel endothelial cells in a mammalian organism;

Group VIII: Claims 73-76, drawn to a method of inhibiting proliferation of blood vessel endothelial cells in a human organism having a disease characterized by expression of Flt4 tyrosine kinase (Flt4) in vascular endothelial cells;

Group IX: Claims 78-80, drawn to a method of inhibiting proliferation of endothelial cells in a human organism having a breast carcinoma characterized by expression of Flt4 tyrosine kinase (Flt4) in vascular endothelial cells;

Group X: Claims 81-84, drawn to a method of inhibiting genesis of blood vessels in a mammalian organism having a disease characterized by expression of Flt4 tyrosine kinase (Flt4) in blood vessels;

Group XI: Claims 85-88, drawn to a method of inhibiting the growth or the metastatic spread of a tumor in a mammalian organism;

Group XII: Claims 89-90, drawn to a method of inhibiting neoplastic cell growth in a human subject; and

Group XIII: Claims 91-93, drawn to a method of inhibiting neoplastic cell growth in a mammalian subject.

The examiner further indicated that upon election of one of the above-mentioned groups, election of one of the following allegedly patentably sub-inventions is also required:

(A) an anti-Flt4 antibody, or a polypeptide comprising an antigen binding fragment thereof;

(B) an anti-VEGF-C antibody, or a polypeptide comprising an antigen binding fragment thereof;

(C) an anti-VEGF-D antibody, or a polypeptide comprising an antigen binding fragment thereof;

(D) a soluble polypeptide comprising a fragment of Flt4, wherein the polypeptide and the fragment are capable of binding to human VEGF-C; and

(E) a polypeptide comprising a Flt4 binding fragment of human prepro-VEGF-C or human propro-VEGF-D conjugated to an antineoplastic agent.

Election

In response to the restriction requirement, applicants hereby provisionally elect Group X (claims 81-84, drawn to a method of inhibiting genesis of blood vessels in a mammalian organism having a disease characterized by expression of Flt4 tyrosine kinase (Flt4) in blood vessels), and "sub-invention" D (directed to a soluble polypeptide comprising a fragment of Flt4, wherein the polypeptide and fragment are capable of binding to human VEGF-C), for continued examination ***with traverse***.

Traversal

A. Review of the PTO's standard and purposes for restriction

The Patent statute only permits restriction of independent and distinct inventions (35 USC 121), which the Patent Office interprets to permit restriction of independent "or" distinct inventions. (MPEP 803, Part I) But such restriction is proper only if there would be a "serious burden" on the examiner in the absence of the restriction. (MPEP 803; 808.02)There is no harm whatsoever in examining multiple independent or distinct inventions in one application. (MPEP 805)

Importantly, the MPEP contains numerous cautions to the effect that restriction practice is not merely a burden-easing tool to be used by examiners when examiners see fit to do so. For example, Section 803 ("Restriction – When Proper") states, "an application may properly be restricted to one of two or more claimed inventions only if they are able to support separate patents and they are either independent or distinct." Section 803.01 states, in all capital letters, "IT STILL REMAINS IMPORTANT FROM THE STANDPOINT OF THE PUBLIC INTEREST THAT NO REQUIREMENTS BE MADE WHICH MIGHT RESULT IN THE ISSUANCE OF TWO PATENTS FOR THE SAME INVENTION." Similarly, Section 804.01 states, "This apparent nullification of double

patenting as a ground of rejection or invalidity in such cases imposes a heavy burden on the Office to guard against erroneous requirements for restrictions where the claims define essentially the same invention in different language and which, if acquiesced in, might result in the issuance of several patents for the same invention." Where inventions are related as disclosed but are not distinct as claimed, restriction is never proper. (MPEP 806; MPEP 808.02) "[I]t is imperative the requirement should never be made where related inventions as claimed are not distinct." (Id.)

In view of the foregoing, the MPEP instructs that "Examiners must provide reasons and/or examples to support conclusions . . . ." (MPEP 803, Part II) See also MPEP 808.01 ("The particular reasons relied on by the examiner for holding that the inventions as claimed are either independent or distinct should be concisely stated. A mere statement of conclusion is inadequate. The reasons upon which the conclusion is based should be given. For example, relative to a combination and a subcombination thereof, the examiner should point out the reasons why he or she considers the subcombination to have utility by itself or in other combinations, and why he or she considers that the combination as claimed does not require the particulars of the subcombination as claimed. Each relationship of claimed inventions should be similarly treated and the reasons for the conclusions of distinctness or independence set forth.") "The examiner must provide a clear and detailed record of the restriction requirement to provide a clear demarcation between restricted inventions so that it can be determined whether inventions claimed in a continuing application are consonant with the restriction requirement and therefore subject to the prohibition against double patenting rejections under 35 U.S.C. 121." (MPEP 814)

B. The current restriction is improper on its face

The examiner has failed to provide a clear and detailed demarcation between restriction groups. This is self-evident from comparison of, e.g., groups III and IV, which are described in *exactly the same way* ("a method of inhibiting neoplastic cell growth in a mammalian subject"). Additionally, the examiner has indicated that claims 55 and 58-60 are in both Groups. It is unclear how claims can be distinct from themselves, especially when the alleged demarcation is not a demarcation. It should be noted that Groups III and IV are also classified in the same class/subclass as the elected Group.

There are many other examples of allegedly distinct groups which, while defined by slightly varying words, are equally difficult to separate by any lines of "clear demarcation." (See, for example, the aforementioned Groups III and IV, compared to, e.g., Group VI, which is directed to a method for treating a neoplastic disorder, or Group XII, directed to a method of inhibiting neoplastic cell growth in a human, or Group XIII, directed to a method of inhibiting neoplastic cell growth in a mammalian subject.) It will be readily apparent that a claim to treating a neoplastic disorder in a human could fall within all of these groups (humans are mammals), and other groups, and it would be impossible to properly assign such a claim to one group.

A related, fundamental defect with the restriction is a total failure to provide reasons for restriction. Instead of explaining how any single group is distinct from any other group, as directed by the MPEP, the restriction merely contains a one sentence conclusion ("The methods of groups I-XIII can be shown to be distinct as they each have different starting materials, method steps, and/or goals.") This is precisely the conclusory treatment of that is prohibited by the MPEP, as quoted above.

It appears that the actual basis for restriction was whether one claim was dependent from another or independent. However, there is no justification, in the provisions for analyzing patentable distinctness, for such a mechanical analysis.

Because the Patent Office has failed to provide demarcations between the groups or explain how the groups are allegedly distinct from each other, the restriction requirement is improper, and moreover, it is exceedingly difficult to traverse on the merits. (One cannot rebut a *prima facie* case that has not been set forth.) Notwithstanding, the Applicants identify some of the other reasons for removing the restriction requirement below.

C. Relatedness of all other groups to elected Group X

Group X is directed to "a method of inhibiting genesis of blood vessels in a mammalian organism having a disease characterized by expression of Flt4 tyrosine kinase in blood vessels. Independent claim 81 in that group includes a step of administering to a mammalian organism (having a disease characterized by expression of Flt4 in blood vessels)

a composition that comprises an inhibitor of the binding between Flt4 and an Flt4 ligand. A Markush group of five classes of inhibitors is recited in the claim.

All of the other groups have varying degrees of relatedness to Group X and its claims. For example, antagonizing or inhibiting Flt4 function is a recurrent theme in the groups. Administering an inhibitor of the binding between Flt4 and its ligands is a recurrent theme in the groups. The Markush group of inhibitors recited in claim 81 is a recurrent theme in the claims of multiple groups. Flt4 expression in blood vessels (in the organism to be treated), and more particularly in the endothelia of the blood vessels, is a recurrent theme in multiple groups. The existence of neoplastic cell growth or a neoplastic disorder generally, or a breast carcinoma specifically, is a recurrent theme in multiple groups.

In order for any restriction to be maintained, it is incumbent upon the Patent Office to identify clear lines of demarcation between the groups, notwithstanding their relatedness, and define groups that maintain such demarcation. Moreover, demarcation alone is insufficient. The Patent Office also must provide reasons for concluding that the groups, so demarked, are distinct.

In the event that such restriction is imposed, the Applicants request that it be made non-final, to permit a traversal of the reasons for restriction.

D. No serious burden

Moreover, applicants request that the restriction requirement be reconsidered because the examiner has not shown that a serious burden would be required to examine all of the claims. M.P.E.P. § 803 provides, "If the search and examination of an application can be made without serious burden, the Examiner **must** examine it on the merits, even though it includes claims to distinct or independent inventions." (*Emphasis added.)* Even a *prima facie* showing of burden (e.g., by appropriate explanation of separate classification, or separate status in the art, or a different field of search as defined in MPEP § 803.02, can be rebutted by appropriate showings or evidence by the applicant.

There would be no serious burden examining all of the claims in this application at one time. Compelling evidence of this is the fact that the prior examiner

examined similar claims (and more claims) to the point of allowance in the parent application. (See Remarks section of the Applicant's preliminary amendment in this case, which correlates pending claims in this case with claims that were examined and allowed in USSN 09/169,079.) The current claims (which contain limitations relating to blood vessel expression of Flt4), are believed to be entitled to a longer patent term than the claims which were permitted to issue in the parent case, by virtue of a later priority claim. However, these claims do not pose a serious burden of examination in one application. Moreover, the relatedness, discussed above, indicates that the search for each method claims will be similar.

Likewise, the allowed claims in the parent application contain limitations directed to a variety of different Flt4 inhibitors. No serious burden was found in examining claims that recite more than one inhibitor – the second "subgroup" (A-E) prong of the restriction requirement.

Conclusion

For the foregoing reasons, applicants request reconsideration and withdrawal of the restriction requirement. Should the examiner have any questions or comments regarding this response or the application, the examiner is invited to contact the undersigned agent or David A. Gass, attorney for applicants, at the number indicated below.

Dated: October 17, 2006

Respectfully submitted,

By Jeanne M. Brashear

Jeanne M. Brashear
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Agent for Applicant

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a) FY 2006 (Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).)	Docket Number (Optional) 28967/34891.1
Application Number 10/774,802-Conf. #9059	Filed February 9, 2004
For FLT4 (VEGFR-3) AS A TARGET FOR TUMOR IMAGING AND ANTI-TUMOR THERAPY	
Art Unit 1646	Examiner Not Yet Assigned

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	
[x]	One month (37 CFR 1.17(a)(1))	$120	$60	$ 60.00
[ ]	Two months (37 CFR 1.17(a)(2))	$450	$225	$
[ ]	Three months (37 CFR 1.17(a)(3))	$1020	$510	$
[ ]	Four months (37 CFR 1.17(a)(4))	$1590	$795	$
[ ]	Five months (37 CFR 1.17(a)(5))	$2160	$1080	$

[x] Applicant claims small entity status. See 37 CFR 1.27.

[ ] A check in the amount of the fee is enclosed.

[ ] Payment by credit card. Form PTO-2038 is attached.

[x] The Director has already been authorized to charge fees in this application to a Deposit Account.

[x] The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 13-2855 . I have enclosed a duplicate copy of this sheet.

I am the

[ ] applicant/inventor.

[ ] assignee of record of the entire interest. See 37 CFR 3.71. Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96).

[x] attorney or agent of record. Registration Number 56,301

[ ] attorney or agent under 37 CFR 1.34. Registration number if acting under 37 CFR 1.34 _____ .

Jeanne M. Brashear
Signature

October 17, 2006
Date

Jeanne M. Brashear
Typed or printed name

(312) 474-6300
Telephone Number

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below.

[ ] Total of 1 forms are submitted.

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted by facsimile to the Patent and Trademark Office, facsimile no. (571) 273-8300, on the date shown below.

Dated: October 17, 2006 Signature: Jeanne M. Brashear (Jeanne M. Brashear)

Office Action Summary	Application No.	Applicant(s)
	10/774,802	ALITALO, KARI
	Examiner	Art Unit
	Ian Dang	1647

-- *The MAILING DATE of this communication appears on the cover sheet with the correspondence address* --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *23 October 2006*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *43-93* is/are pending in the application.

4a) Of the above claim(s) *43-80 and 85-93* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *81-84* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☒ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Election/Restrictions

Applicant's election with traverse of Group X, claims 81-84 in the communication filed on 10/23/2006 is acknowledged. Applicant has further elected sub-invention D, which is directed to a soluble polypeptide comprising a fragment of Flt4, wherein the polypeptide and fragment are capable of binding to a human VEGF-C. The traversal is on the ground that Group X is related to all the other groups and there is no undue burden to search the other groups. This is not found persuasive for the following reasons:

Applicant's attention is directed to MPEP 808.02 which states that "Where the related inventions as claimed are shown to be distinct under the criteria of MPEP 806.05(c-I), the examiner, in order to establish reasons for insisting upon restriction, must show by appropriate explanation one of the following: (A) Separate classification thereof: (B) A separate status in the art when they are classifiable together; (C) A different field of search." As set forth in the Restriction requirement, the separate classification established for each Group demonstrates that each distinct Group has attained recognition in the art as a separate subject for inventive effort, and also a separate field of search. Thus, the Restriction requirement is proper.

Applicant argues that no burden is placed on the examiner to consider all claims. As discussed above, the separate classification established for each Group demonstrates that each distinct Group requires a separate field of search, and a search of one Group would not reveal art on the other Groups, thus imposing a burden on the examiner. Furthermore, each group requires a non-coextensive sequence and non-patent literature search.

The requirement is still deemed proper and is therefore made FINAL. Claims 43-80 and 85-93 are withdrawn from further consideration pursuant to 37 CFR 1.142(b).

Claims 81-84 are pending and under examination.

Specification

When there are benefit claims to multiple prior nonprovisional applications (e.g., a string of prior nonprovisional applications), the relationship must include identification of each nonprovisional application as either a continuation, divisional, or continuation-in-part application of a specific prior nonprovisional application for which a benefit is claimed. See United States Patent and Trademark Office OG Notices: 1268 OG 89 (18 March 2003).

Further, the specification on page 1, paragraph 1, should be amended to reflect the status of priority applications USSN 09/929,612 (now US Patent No. 6,706,870), USSN 08/250,846 (now US Patent No. 6,562,333), USSN 08/177,747 (abandoned), and USSN 08/077,203 (abandoned).

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 81-84 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for a method of inhibiting lymphangiogenesis in a mammalian organism having a disease characterized by expression of Flt4 tyrosine kinase in blood vessels, does not reasonably provide enablement for a method of inhibiting genesis of blood vessels in a mammalian organism having a disease characterized by expression of Flt4 tyrosine kinase (Flt4) in blood vessels. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make/use the invention commensurate in scope with these claims.

In In re Wands, 8USPQ2d, 1400 (CAFC 1988) page 1404, the factors to be considered in determining whether a disclosure would require undue experimentation include: (1) Nature of the invention, (2) the state of the prior art, (3) the predictability or lack thereof in the art, (4) the amount of direction or guidance present, (5) the presence or absence of working examples, (6) the breath of the claims, (7) the quantity of experimentation needed, (8) relative skill of those in the art.

Nature of the invention and breath of the claims

The claims are drawn to a method of inhibiting genesis of blood vessels in a mammalian organism having a disease characterized by expression of Flt4 tyrosine kinase (Flt4) in blood vessels. The invention is broad because the recitation of claims 81-84 encompasses the formation of old and new blood vessels from existing vessels by angiogenesis and new blood vessels by vasculogenesis. These 2 types of biological processes have distinct signals and are induced under different conditions.

Unpredictability and state of the art

The state of the art for the role of Flt4 in the formation of the lymphatic vasculature is well established. Alitalo et al. (2005) teach the mechanisms for lymphangiogenesis in development and human disease in a recent review article.

However, the role of Flt4 in the formation of blood vessel is not predictable. Tammela et al. (2005) teach that Flt4 or VEGFR-3 is present in all endothelial during development but in the adult it becomes restricted to lymphatic endothelial cells and certain fenestrated blood vascular endothelial cells. VEGFR-3 is upregulated on blood vascular endothelial cells in pathologic conditions, such as vascular tumors and in the periphery of solid tumors (page 556, column 1,

1st paragraph). While the role of Flt4 on existing endothelial is well studies, the art is silent in the role of Flt4 in the genesis of blood vessels.

In addition, the VEGF-C, the ligand for Flt4, is not involved with the formation of new blood vessels from existing blood vessels. Ferrara et al. (1999) teach that a K14-driven VEGF-C transgene induced lymphangiogenesis but no angionenesis in mouse skin, and recombinant VEGF-C also stimulated lymphatic vessel hyperplasia in mature chick chorioallantoic membrane (page 1362, column 2, 2nd paragraph).

In view of these teachings, a method of inhibiting genesis of blood vessels in a mammalian organism having a disease characterized by expression of Flt4 tyrosine kinase (Flt4) in blood vessels is not predictable.

The amount of direction or guidance present

Applicants' disclosure is limited to the identification of genes responsible for affecting sleep in flies. The specification does not provide guidance or direction regarding how can Flt4 is involved in the formation of blood vessels and how the inhibition for the genesis of blood vessels can be mediated through Flt4. Since Applicants have not linked the expression of Flt4 with the formation of blood vessels, Flt4 may not have any effects on the formation of blood vessels.

Working Examples

Although Applicants have provided several examples for a method of inhibiting the formation of lymphatic system, the specification does not provide any examples for a method of inhibiting genesis of blood vessels in a mammalian organism having a disease characterized by expression of Flt4 tyrosine kinase (Flt4) in blood vessels. Flt4 is a critical protein for lymphangiogenesis, but its role for the formation of blood vessels in angiogenesis or

vasculogenesis has not been illustrated in any examples in the specification of the instant application.

The quantity of experimentation needed

Because the claims are broadly drawn to a method of inhibiting genesis of blood vessels in a mammalian organism having a disease characterized by expression of Flt4 tyrosine kinase (Flt4) in blood vessels, and because Applicant's disclosure does not contain sufficient teachings to overcome the unpredictability taught in the art, it would require undue experimentation by one of skill in the art to be able to practice the invention commensurate in scope with the claims.

Conclusion

No claims are allowed.

Information

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Ian Dang whose telephone number is (571) 272-5014. The examiner can normally be reached on Monday-Friday from 9am to 5pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Brenda Brumback can be reached on (571) 272-0961. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

Ian Dang
Patent Examiner
Art Unit 1647
November 8, 2006

BRENDA BRUMBACK
SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 1600

Docket No.: 28967/34891.1
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Kari Alitalo	Confirmation No.: 9059
Serial No.: 10/774,802	Art Unit: 1647
Filed: February 9, 2004	
For: Flt4 (VEGFR-3) as a Target for Tumor and Anti-Tumor Therapy	Examiner: Ian D. Dang

DECLARATION UNDER 37 C.F.R. § 1.132 OF DR. KARI ALITALO

I, Dr. Kari Alitalo, do hereby declare and state as follows:

1. I am an inventor of the invention claimed in the above-referenced patent application (hereinafter, "the patent application"). I currently hold the position of Research Professor of the Finnish Academy of Sciences and am the lead scientist in a laboratory in Finland staffed with post-doctoral, graduate student and other researchers. My laboratory also collaborates to varying degrees with other scientists from time to time.

2. The invention is directed, in part, to a method of inhibiting genesis of blood vessels in a mammalian organism using an inhibitor of the binding of an Flt4 ligand protein to Flt4. Exemplary claims defining the invention are appended hereto as Exhibit A.

3. I understand that the application currently stands rejected, in part because the Examiner has questioned whether the invention works as described. The application discloses that VEGFR-3/PAL-E positive vessels are detectable in intraductal carcinomas, and describes the use of an agent that blocks VEGF-C or VEGF-D stimulation of VEGFR-3 (or VEGFR-2) as a treatment incanceres characterized by VEGFR-3 positive blood vessels. Subsequent to the filing of the patent application, I have been involved in further research which confirms that inhibition of VEGFR-3-ligand binding inhibits blood vessel growth *in vivo* and correspondingly inhibits primary tumor growth. One purpose of this declaration is to summarize some of these

experiments. I also am familiar with a study, by another laboratory after the application was filed, related to this topic, and I am familiar with certain publications cited by the Examiner in the rejection of the application. Another purpose of the declaration is to comment on these publications and their relevance to the application.

4. Laakkonen et al., *Cancer Res.*, 67:593-599, 2007 (hereinafter "Laakkonen", submitted herewith as Exhibit B), of which I am a named author, describes that VEGFR-3 is involved in tumor angiogenesis and growth. Co-authors of the Laakkonen publication include Laakkonen P, Waltari M, Holopainen T, Takahashi T, Pytowski B, Steiner P, Hicklin D, Persaud K, Tonra JR, and Witte L. We designed the experiments described in Laakkonen et al. to determine if an inhibitor that prevented ligand binding to VEGFR-3 (Flt-4) could inhibit primary tumor growth and/or tumor angiogenesis *in vivo*. Nude mice and SCID mice bearing human-derived tumors were employed. An anti-VEFGR-3 antibody that blocks ligand (VEGF-C or –D) binding to VEGFR-3 inhibited the growth of *several different* tumor cell lines in the nude mice, as well as inhibited primary tumors. Importantly, histological analysis of the mice, using antibodies for blood vascular endothelial cell markers, revealed that the anti-VEGFR-3 antibody therapy had *significantly decreased blood vessel density*, in comparison with blood vessel density in tumors of mice that were treated with a control antibody. We also performed histological studies using antibodies against VEGFR-3, the results of which demonstrated that the tumor blood vessels expressed VEGFR-3. Additional analysis showed evidence of hypoxia and necrosis in the tumors treated with the anti-VEGFR-3 antibody. Collectively, the data indicates that anti-Flt4 (anti-VEGFR-3) therapy inhibits tumor growth through a mechanism that involves inhibition of angiogenesis.

5. I hereby confirm that the Laakkonen paper is an accurate description of experiments in which I have participated with others in my laboratory or my collaborators, and the results that we obtained. I incorporate the text of the paper into this declaration by reference.

6. Kubo et al., (Blood, 96:546-553, 2000, attached hereto as Exhibit C), of which I am a co-author, reported that the rat anti-VEGFR-3 monoclonal antibody AFL4 inhibited tumor xenograft growth in mice by compromising the integrity of tumor blood vessels, leading to microhemorrhages. When our laboratory tested the AFL4 antibody, it inhibited neither ligand binding nor phosphorylation of VEGFR-3, nor blocked VEGF-C-stimulated cell proliferation.

Thus, the *in vivo* effects of the AFL4 antibody are mediated by a mechanism other than the direct inhibition of ligand binding. We discussed the Kubo et al. reference in the Laakkonen publication.

6. I have reviewed the published studies of yet another publication pertaining to the role of VEGFR-3 in anti-angiogenic therapy. Roberts et al. (Cancer Res., 66:2650-2657, 2006, attached hereto as Exhibit D), employed receptor-specific antagonist antibodies in an orthotopic spontaneous breast cancer metastasis model to provide direct evidence for the key role of VEGFR-3 activation in metastasis. Nude mice bearing human-derived tumors were used. An anti-VEGFR-3 antibody inhibited the growth of a breast carcinoma cell line in the nude mice. Histological analysis of the mice, using antibodies for blood vascular endothelial cell markers, revealed that treatment with the anti-VEGFR-3 antibody reduced blood vessel densities and decreased the size of tumor blood vessels compared to controls. Histological studies were performed using antibodies against VEGFR-3, the results of which demonstrated that the tumor blood vessels expressed VEGFR-3. The results of this study are consistent with the teachings in the application that administration of an inhibitor of ligand-mediated signaling of VEGFR-3 can successfully inhibit tumor growth and angiogenesis.

7. I am a co-author of the Ferrera et al. document cited by the Examiner in the Office action. I understand that page 1362 of Ferrara et al. was cited by the Examiner as evidence that VEGF-C is not involved with the formation of new blood vessels from existing blood vessels. Ferrera et al. review the clinical applications of angiogenic growth factors and their inhibitors. In particular, at page 1362, Ferrera et al. review the findings of Jeltsch et al. (Science, 276:1423-1425, 1997, set forth in Appendix E) and Oh et al. (Dev. Biol., 188:96-109, 1997, Set forth in Appendix F). I am named as a co-author of both the Jeltsch et al. and Oh et al. publications. Jeltsch et al. report that overexpression of VEGF-C in the skin of transgenic mice resulted in lymphatic, but not vascular, endothelial proliferation and vessel enlargement. Oh et al. report that recombinant VEGF-C stimulated lymphatic vessel hyperplasia in mature chick chorioallantoic membrane. Neither Jeltsch et al. nor Oh et al. utilized a model for a disease characterized by *Flt4 expression in blood vessels* in their experiments. Although expression of Flt4 in healthy adult tissues is largely restricted to lymphatics, the present patent application is based in part on the discovery that Flt4 is expressed in the blood vasculature of certain cancers,

and that ligand-mediated signaling of Flt4 results in tumor angiogenesis. In view of the different animal systems employed it is not inconsistent that our studies reported in Jeltsch et al. and Oh et al. observed lymphatic effects but not blood vessel effects. Thus, it is true that the blood vessel effects were not predictable based on these prior studies. However, the fact that these prior studies would not predict the current invention is a separate question from whether the present invention, as taught in the application, predictably works. The studies discussed in the preceding paragraphs of this declaration show that the invention works.

8. I am a co-author of the Tammela et al. document cited by the Examiner in the Office action. Tammela et al. report that VEGFR-3 becomes restricted to lymphatic endothelial cells in adults, and further states that VEGFR-3 is also up-regulated on blood vascular endothelial cells in pathologic conditions, such as vascular tumors and in the periphery of solid tumors. Thus, the teachings of Tammela et al. are consistent with the teachings of the present application and confirm that VEGFR-3 is expressed in the blood vasculature of certain tumors. In fact, the Tammela et al. publication harmonizes the observations involving Flt4/VEGFR-3 expression in healthy adult tissues versus pathogenic ones, insofar as understanding why the present invention is exciting and effective.

9. I further declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both (18 U.S.C. § 1001), and may jeopardize the validity of the application or any patent issuing thereon.

Date 15.3.2007

Dr. Kari Alitalo

EXHIBIT A

1.-42. (Canceled)

43. (Withdrawn) A method of inhibiting Flt4 receptor tyrosine kinase (Flt4) function in a mammalian organism with a neoplastic disease, comprising the step of administering to said mammalian organism a composition,

wherein said neoplastic disease is a breast carcinoma characterized by expression of Flt4 in vascular endothelial cells,

wherein said composition comprises an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in blood vascular endothelial cells of said organism, and

wherein said composition is administered in an amount effective to inhibit Flt4-mediated proliferation of said vascular endothelial cells, thereby inhibiting Flt4-mediated proliferation of said vascular endothelial cells.

44. (Withdrawn) A method according to claim 43 wherein said organism is human.

45. (Withdrawn) A method according to claim 43 wherein said inhibitor comprises a polypeptide selected from the group consisting of:

(a) an anti-Flt4 antibody or a polypeptide comprising an antigen binding fragment thereof;

(b) an anti-VEGF-C antibody or a polypeptide comprising an antigen binding fragment thereof;

(c) an anti-VEGF-D antibody or a polypeptide comprising an antigen binding fragment thereof;

(d) a soluble polypeptide comprising a fragment of Flt4, wherein the polypeptide and the fragment are capable of binding to human VEGF-C (SEQ ID NO: 21); and

(e) a polypeptide comprising an Flt4 binding fragment of human prepro-VEGF-C (SEQ ID NO: 21) or human prepro-VEGF-D (SEQ ID NO: 22) conjugated to an antineoplastic agent.

46. (Withdrawn) A method according to claim 43, wherein the inhibitor comprises a bispecific antibody, or fragment thereof, wherein said antibody or fragment specifically binds Flt4 and specifically binds a blood vascular endothelial marker antigen.

47. (Withdrawn) A method according to claim 46 wherein said blood vascular endothelial marker antigen is selected from the group consisting of PAL-E, VEGFR-1, and VEGFR-2.

48. (Withdrawn) A method according to claim 46 wherein said inhibitor further comprises an anti-neoplastic agent conjugated to said bispecific antibody.

49. (Withdrawn) A method for antagonizing the function of Flt4 receptor tyrosine kinase (Flt4) in an organism, comprising a step of administering to the organism a composition comprising a polypeptide and a pharmaceutically acceptable carrier, wherein the polypeptide is selected from the group consisting of:

(a) an anti-Flt4 antibody or a polypeptide comprising an antigen binding fragment thereof;

(b) an anti-VEGF-C antibody or a polypeptide comprising an antigen binding fragment thereof;

(c) an anti-VEGF-D antibody or a polypeptide comprising an antigen binding fragment thereof;

(d) a soluble polypeptide comprising a fragment of Flt4, wherein the polypeptide and the fragment are capable of binding to human VEGF-C (SEQ ID NO: 21); and

(e) a polypeptide comprising an Flt4 binding fragment of human prepro-VEGF-C (SEQ ID NO: 21) or human prepro-VEGF-D (SEQ ID NO: 22) conjugated to an antineoplastic agent; and

wherein the organism has a neoplastic disorder characterized by blood vessels comprising endothelial cells that express Flt4.

50. (Withdrawn) A method for antagonizing the function of Flt4 receptor tyrosine kinase (Flt4) in an organism, comprising a step of administration to the organism a composition comprising a bispecific antibody, or fragment thereof,

wherein said antibody or fragment specifically binds Flt4 and specifically binds a blood vascular endothelial marker antigen; and

wherein said organism has a neoplastic disorder characterized by blood vessels comprising endothelial cells that express Flt4.

51. (Withdrawn) A method according to claim 49 or 50, wherein the organism is human.

52. (Withdrawn) A method according to claim 50, wherein said composition further comprises an anti-neoplastic agent conjugated to said antibody or antibody fragment.

53. (Withdrawn) A method of inhibiting neoplastic cell growth in a mammalian subject, comprising steps of:

(a) screening a mammalian subject to identify a neoplastic disorder characterized by blood vessels that comprise endothelial cells that express Flt4; and

(b) administering a composition to a mammalian subject identified according to step (a) as having a neoplastic disorder characterized by cells expressing Flt4, said composition comprising an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in cells of said subject, thereby inhibiting Flt4-mediated proliferation of said Flt4-expressing cells, wherein said inhibitor comprises a polypeptide selected from the group consisting of:

(i) an anti-Flt4 antibody or a polypeptide comprising an antigen binding fragment thereof;

(ii) an anti-VEGF-C antibody or a polypeptide comprising an antigen binding fragment thereof;

(iii) an anti-VEGF-D antibody or a polypeptide comprising an antigen binding fragment thereof; and

(iv) a soluble polypeptide comprising a fragment of Flt4, wherein the polypeptide and the fragment are capable of binding to human VEGF-C (SEQ ID NO: 21);

(v) a polypeptide comprising an Flt4 binding fragment of human prepro-VEGF-C (SEQ ID NO: 21) or human prepro-VEGF-D (SEQ ID NO: 22) conjugated to an antineoplastic agent.

54. (Withdrawn) A method of inhibiting neoplastic cell growth in a mammalian subject, comprising steps of:

(a) screening a mammalian subject to identify a neoplastic disorder characterized by blood vessels that comprise endothelial cells that express Flt4; and

(b) administering a composition to a mammalian subject identified according to step (a) as having a neoplastic disorder characterized by cells expressing Flt4, said composition comprising an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in cells of said subject, thereby inhibiting Flt4-mediated proliferation of said Flt4-expressing cells,

wherein said inhibitor comprises a bispecific antibody, or fragment thereof, wherein said antibody or fragment specifically binds Flt4 and specifically binds a blood vascular endothelial marker antigen.

55. (Withdrawn) A method according to claims 53 or 54, wherein the mammalian subject is human.

56. (Withdrawn) A method according to claim 54 wherein said blood vascular endothelial marker antigen is selected from the group consisting of PAL-E, VEGFR-1, and VEGFR-2.

57. (Withdrawn) A method according to claim 54 wherein said inhibitor further comprises an anti-neoplastic agent conjugated to said bispecific antibody.

58. (Withdrawn) A method according to claims 53 or 54 wherein the screening step comprises:

(a) contacting tissue from the mammalian subject with a composition comprising an antibody or antibody fragment that specifically binds Flt4;

(b) detecting said antibody or antibody fragment bound to cells in said tissue; and

(c) screening for a neoplastic disorder characterized by blod vessels that comprise endothelial cells that express Flt4 from the quantity or distribution of said antibody bound to cells in said tissue.

59. (Withdrawn) A method according to claim 58 wherein in said screening step, the detection of said antibody or antibody fragment bound to blood vessel endothelial cells is correlated with the presence of a neoplastic disease.

60. (Withdrawn) The method according to claim 59 wherein said tissue comprises mammary tissue.

61. (Withdrawn) A method of treating a mammal having breast cancer characterized by blood vessel endothelial cells that express Flt4 tyrosine kinase (Flt4), comprising a step of administering to said mammal a composition, said composition comprising an inhibitor of binding between Flt4 ligand protein and Flt4 expressed in cells of said organism, thereby inhibiting Flt4 function, wherein the inhibitor comprises a member selected from the group consisting of:

(a) an anti-Flt4 antibody or a polypeptide comprising an antigen binding fragment thereof;

(b) an anti-VEGF-C antibody or a polypeptide comprising an antigen binding fragment thereof;

(c) an anti-VEGF-D antibody or a polypeptide comprising an antigen binding fragment thereof;

(d) a soluble polypeptide comprising a fragment of Flt4, wherein the polypeptide and the fragment are capable of binding to human VEGF-C (SEQ ID NO: 21); and

(e) a polypeptide comprising an Flt4 binding fragment of human prepro-VEGF-C (SEQ ID NO: 21) or human prepro-VEGF-D (SEQ ID NO: 22) conjugated to an antineoplastic agent.

62. (Withdrawn) A method of treating a mammal having breast cancer characterized by blood vessel endothelial cells that express Flt4 tyrosine kinase (Flt4), comprising a step of administering to said mammal a composition, said composition comprising an inhibitor of binding between Flt4 ligand protein and Flt4 expressed in cells of said organism, thereby inhibiting Flt4 function,

wherein the inhibitor comprises a bispecific antibody, or fragment thereof, wherein said antibody or fragment specifically binds Flt4 and specifically binds a blood vascular endothelial marker antigen.

63. (Withdrawn) A method according to claims 61 or 62 wherein said mammal is human.

64. (Withdrawn) A method according to claim 61 or 62 comprising a screening step preceding the administering step, wherein the screening step comprises screening a human to identify breast cancer characterized by blood vessel endothelial cells expressing Flt4; and

wherein the administering step comprises administering the composition to a human identified by the screening step as having breast cancer characterized by blood vessel endothelial cells expressing Flt4.

65. (Withdrawn) A method for treating a neoplastic disorder in a mammalian subject, comprising the steps of:

(a) screening a mammalian subject to identify a neoplastic disorder characterized by blood vessel endothelial cells expressing Flt4 receptor tyrosine kinase (Flt4); and

(b) administering a composition to a mammalian organism identified according to step (a) as having a neoplastic disorder characterized by blood vessel endothelial cells expressing Flt4, to inhibit Flt4 mediated proliferation of said Flt4-expressing cells,

wherein the composition comprises a means for inhibiting Flt4 function in mixture with a pharmaceutically acceptable diluent, adjuvant, or carrier.

66. (Withdrawn) A method according to claim 65, wherein the mammalian subject is human.

67. (Withdrawn) A method according to claim 66, wherein the means for inhibiting comprises a member selected from the group consisting of:

(a) an anti-Flt4 antibody or a polypeptide comprising an antigen binding fragment thereof;

(b) an anti-VEGF-C antibody or a polypeptide comprising an antigen binding fragment thereof;

(c) an anti-VEGF-D antibody or a polypeptide comprising an antigen binding fragment thereof;

(d) a soluble polypeptide comprising a fragment of Flt4, wherein the polypeptide and the fragment are capable of binding to human VEGF-C (SEQ ID NO: 21); and

(e) a polypeptide comprising an Flt4 binding fragment of human prepro-VEGF-C (SEQ ID NO: 21) or human prepro-VEGF-D (SEQ ID NO: 22) conjugated to an antineoplastic agent.

68. (Withdrawn) A method according to claim 66, wherein the means for inhibiting comprises a bispecific antibody, or fragment thereof, wherein said antibody or

fragment specifically binds Flt4 and specifically binds a blood vascular endothelial marker antigen.

69. (Withdrawn) A method according to claim 68 wherein said blood vascular endothelial marker antigen is selected from the group consisting of PAL-E, VEGFR-1, and VEGFR-2.

70. (Withdrawn) A method according to claim 68 wherein the means for inhibiting further comprises an anti-neoplastic agent conjugated to said bispecific antibody.

71. (Withdrawn) A method of inhibiting proliferation of blood vessel endothelial cells in a mammalian organism having a disease characterized by expression of Flt4 tyrosine kinase (Flt4) in blood vessel endothelial cells, comprising the step of administering to said mammalian organism a composition, said composition comprising an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in blood vessel endothelial cells of said organism, thereby inhibiting Flt4-mediated proliferation of the blood vessel endothelial cells, wherein said inhibitor comprises a polypeptide selected from the group consisting of:

(a) an anti-Flt4 antibody or a polypeptide comprising an antigen binding fragment thereof;

(b) an anti-VEGF-C antibody or a polypeptide comprising an antigen binding fragment thereof;

(c) an anti-VEGF-D antibody or a polypeptide comprising an antigen binding fragment thereof;

(d) a soluble polypeptide comprising a fragment of Flt4, wherein the polypeptide and the fragment are capable of binding to human VEGF-C (SEQ ID NO: 21); and

(e) a polypeptide comprising an Flt4 binding fragment of human prepro-VEGF-C (SEQ ID NO: 21) or human prepro-VEGF-D (SEQ ID NO: 22) conjugated to an antineoplastic agent.

72. (Withdrawn) A method of inhibiting proliferation of blood vessel endothelial cells in a mammalian organism having a disease characterized by expression of Flt4 tyrosine kinase (Flt4) in blood vessel endothelial cells, comprising the step of administering to said mammalian organism a composition, said composition comprising an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in blood vessel endothelial cells of said organism, thereby inhibiting Flt4-mediated proliferation of the blood vessel endothelial cells,

wherein the inhibitor comprises a bispecific antibody, or fragment thereof, wherein said antibody or fragment specifically binds Flt4 and specifically binds a blood vascular endothelial marker antigen.

73. (Withdrawn) A method of inhibiting proliferation of endothelial cells in a human organism having a disease characterized by expression of Flt4 tyrosine kinase (Flt4) in endothelial cells, comprising the step of administering to said human organism a composition, said composition comprising an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in endothelial cells of said organism, thereby inhibiting Flt4-mediated proliferation of the cells, wherein the inhibitor comprises a bispecific antibody, or fragment thereof, wherein said antibody or fragment specifically binds Flt4 and specifically binds a blood vascular endothelial marker antigen.

74. (Withdrawn) A method according to claim 72 or 73, wherein said blood vascular endothelial marker antigen is selected from the group consisting of PAL-E, VEGFR-1, and VEGFR-2.

75. (Withdrawn) A method according to claim 72 or 73, wherein said inhibitor further comprises an anti-neoplastic agent conjugated to said bispecific antibody.

76. (Withdrawn) A method according to claim 72 or 73, wherein the disease is a tumor characterized by blood vessels with endothelial cells expressing Flt4.

77. (Withdrawn) A method according to claim 66, wherein the disease is a breast carcinoma characterized by expression of Flt4 in vascular endothelial cells.

78. (Withdrawn) A method of inhibiting proliferation of endothelial cells in a human organism having a breast carcinoma characterized by expression of Flt4 tyrosine kinase (Flt4) in vascular endothelial cells, comprising the step of administering to said human organism a composition, said composition comprising an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in endothelial cells of said organism, thereby inhibiting Flt4-mediated proliferation of the cells, wherein the inhibitor comprises a bispecific antibody, or fragment thereof, wherein said antibody or fragment specifically binds Flt4 and specifically binds a blood vascular endothelial marker antigen.

79. (Withdrawn) A method according to claim 78 wherein said blood vascular endothelial marker antigen is selected from the group consisting of PAL-E, VEGFR-1, and VEGFR-2.

80. (Withdrawn) A method according to claim 78 wherein said inhibitor further comprises an anti-neoplastic agent conjugated to said bispecific antibody.

81. (Previously presented) A method of inhibiting genesis of blood vessels in a mammalian organism having a disease characterized by expression of Flt4 tyrosine kinase (Flt4) in blood vessels, comprising the step of administering to said mammalian organism a composition, said composition comprising an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in cells of said organism, thereby inhibiting Flt4-mediated proliferation of the cells, wherein said inhibitor comprises a member selected from the group consisting of:

(a) an anti-Flt4 antibody or a polypeptide comprising an antigen binding fragment thereof;

(b) an anti-VEGF-C antibody or a polypeptide comprising an antigen binding fragment thereof;

(c) an anti-VEGF-D antibody or a polypeptide comprising an antigen binding fragment thereof;

(d) a soluble polypeptide comprising a fragment of Flt4, wherein the polypeptide and the fragment are capable of binding to human VEGF-C (SEQ ID NO: 21); and

(e) a polypeptide comprising an Flt4 binding fragment of human prepro-VEGF-C (SEQ ID NO: 21) or human prepro-VEGF-D (SEQ ID NO: 22) conjugated to an antineoplastic agent.

82. (Previously presented) A method of inhibiting genesis of blood vessels in a mammalian organism having a disease characterized by expression of Flt4 tyrosine kinase (Flt4) in blood vessels, comprising the step of administering to said mammalian organism a composition, said composition comprising an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in cells of said organism, thereby inhibiting Flt4-mediated proliferation of the cells,

wherein the inhibitor comprises a bispecific antibody, or fragment thereof, wherein said antibody or fragment specifically binds Flt4 and specifically binds a blood vascular endothelial marker antigen.

83. (Previously presented) A method according to claim 81 or 82 wherein said organism is human.

84. (Previously presented) A method according to claim 83, wherein the human has a tumor characterized by blood vessels that express Flt4.

85. (Withdrawn) A method of inhibiting the growth or the metastatic spread of a tumor in a mammalian organism, comprising steps of administering to a mammalian organism a composition that comprises an inhibitor of the binding of an Flt4 ligand protein to Flt4 receptor tyrosine kinase (Flt4) expressed in cells of said organism, wherein the mammalian organism has a tumor characterized by blood and lymphatic vessels that express Flt4, and wherein the composition inhibits proliferation of the blood and lymphatic vessels, thereby inhibiting growth or metastatic spread of the tumor, wherein the inhibitor comprises a member selected from the group consisting of:

(a) an anti-Flt4 antibody or a polypeptide comprising an antigen binding fragment thereof;

(b) an anti-VEGF-C antibody or a polypeptide comprising an antigen binding fragment thereof;

(c) an anti-VEGF-D antibody or a polypeptide comprising an antigen binding fragment thereof;

(d) a soluble polypeptide comprising a fragment of Flt4, wherein the polypeptide and the fragment are capable of binding to human VEGF-C (SEQ ID NO: 21); and

(e) a polypeptide comprising an Flt4 binding fragment of human prepro-VEGF-C (SEQ ID NO: 21) or human prepro-VEGF-D (SEQ ID NO: 22) conjugated to an antineoplastic agent.

86. (Withdrawn) A method of inhibiting the growth or the metastatic spread of a tumor in a mammalian organism, comprising steps of administering to a mammalian organism a composition that comprises an inhibitor of the binding of an Flt4 ligand protein to Flt4 receptor tyrosine kinase (Flt4) expressed in cells of said organism, wherein the mammalian organism has a tumor characterized by blood and lymphatic vessels that express Flt4, and wherein the composition inhibits proliferation of the blood and lymphatic vessels, thereby inhibiting growth or metastatic spread of the tumor,

wherein the inhibitor comprises a bispecific antibody, or fragment thereof, wherein said antibody or fragment specifically binds Flt4 and specifically binds a blood vascular endothelial marker antigen.

87. (Withdrawn) A method according to claim 85 or 86, comprising a diagnosing step prior to the administering step, said diagnosing step comprising identifying a patient having a tumor characterized by blood vessels that express Flt4.

88. (Withdrawn) A method according to claim 85 or 86, comprising a diagnosing step prior to the administering step, said diagnosing step comprising identifying a patient having lymph node metastasis of a tumor, wherein the lymph node comprises cells expressing Flt4.

89. (Withdrawn) A method of inhibiting neoplastic cell growth in a human subject, comprising steps of:

(a) screening a human subject to identify a neoplastic disorder characterized by blood vessels that comprise endothelial cells expressing Flt4 receptor tyrosine kinase (Flt4); and

(b) administering a composition to a human subject identified according to step (a) as having a neoplastic disorder characterized by blood vessels that comprise endothelial cells expressing Flt4, said composition comprising an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in cells of said subject, thereby inhibiting Flt4-mediated proliferation of said Flt4-expressing cells, wherein the inhibitor comprises a member selected from the group consisting of:

(i) an anti-Flt4 antibody or a polypeptide comprising an antigen binding fragment thereof;

(ii) an anti-VEGF-C antibody or a polypeptide comprising an antigen binding fragment thereof;

(iii) an anti-VEGF-D antibody or a polypeptide comprising an antigen binding fragment thereof;

(iv) a soluble polypeptide comprising a fragment of Flt4, wherein the polypeptide and the fragment are capable of binding to human VEGF-C (SEQ ID NO: 21); and

(v) a polypeptide comprising an Flt4 binding fragment of human prepro-VEGF-C (SEQ ID NO: 21) or human prepro-VEGF-D (SEQ ID NO: 22) conjugated to an antineoplastic agent.

90. (Withdrawn) A method of inhibiting neoplastic cell growth in a human subject, comprising steps of:

(a) screening a human subject to identify a neoplastic disorder characterized by blood vessels that comprise endothelial cells expressing Flt4 receptor tyrosine kinase (Flt4); and

(b) administering a composition to a human subject identified according to step (a) as having a neoplastic disorder characterized by blood vessels that comprise endothelial cells expressing Flt4, said composition comprising an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in cells of said subject, thereby inhibiting Flt4-mediated proliferation of said Flt4-expressing cells,

wherein the inhibitor comprises a bispecific antibody, or fragment thereof, wherein said antibody or fragment specifically binds Flt4 and specifically binds a blood vascular endothelial marker antigen.

91. (Withdrawn) A method of inhibiting neoplastic cell growth in a mammalian subject, comprising steps of:

(a) screening a mammalian subject to identify a neoplastic disorder characterized by blood vessel endothelial cells expressing Flt4 receptor tyrosine kinase (Flt4), wherein the screening comprises

(i) contacting tissue from the mammalian subject with a composition comprising an antibody or antibody fragment that specifically binds Flt4;

(ii) detecting said antibody or antibody fragment bound to cells in said tissue; and

(iii) screening for a neoplastic disorder from the quantity or distribution of said antibody or antibody fragment bound to cells in said tissue, wherein the detection of said antibody or antibody fragment bound to blood vessel endothelial cells is correlated with the presence of a neoplastic disease; and

(b) administering a composition to a mammalian subject identified according to step (a) as having a neoplastic disorder characterized by blood vessel endothelial cells expressing

Flt4, said composition comprising an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in cells of said subject, thereby inhibiting Flt4-mediated proliferation of said Flt4-expressing cells, wherein the inhibitor comprises a member selected from the group consisting of:

(i) an anti-Flt4 antibody or a polypeptide comprising an antigen binding fragment thereof;

(ii) an anti-VEGF-C antibody or a polypeptide comprising an antigen binding fragment thereof;

(iii) an anti-VEGF-D antibody or a polypeptide comprising an antigen binding fragment thereof;

(iv) a soluble polypeptide comprising a fragment of Flt4, wherein the polypeptide and the fragment are capable of binding to human VEGF-C (SEQ ID NO: 21); and

(v) a polypeptide comprising an Flt4 binding fragment of human prepro-VEGF-C (SEQ ID NO: 21) or human prepro-VEGF-D (SEQ ID NO: 22) conjugated to an antineoplastic agent.

92. (Withdrawn) A method of inhibiting neoplastic cell growth in a mammalian subject, comprising steps of:

(a) screening a mammalian subject to identify a neoplastic disorder characterized by blood vessel endothelial cells expressing Flt4 receptor tyrosine kinase (Flt4), wherein the screening comprises

(i) contacting tissue from the mammalian subject with a composition comprising an antibody or antibody fragment that specifically binds Flt4;

(ii) detecting said antibody or antibody fragment bound to cells in said tissue; and

(iii) screening for a neoplastic disorder from the quantity or distribution of said antibody or antibody fragment bound to cells in said tissue, wherein the detection of said antibody or antibody fragment bound to blood vessel endothelial cells is correlated with the presence of a neoplastic disease; and

(b) administering a composition to a mammalian subject identified according to step (a) as having a neoplastic disorder characterized by blood vessel endothelial cells expressing Flt4, said composition comprising an inhibitor of the binding of an Flt4 ligand protein to Flt4 expressed in cells of said subject, thereby inhibiting Flt4-mediated proliferation of said Flt4-expressing cells,

wherein the inhibitor comprises a bispecific antibody, or fragment thereof, wherein said antibody or fragment specifically binds Flt4 and specifically binds a blood vascular endothelial marker antigen.

93. (Withdrawn) A method according to claim 91 or 92, wherein said tissue comprises mammary tissue.

Vascular Endothelial Growth Factor Receptor 3 Is Involved in Tumor Angiogenesis and Growth

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Abstract

Vascular endothelial growth factor receptor 3 (VEGFR-3) binds VEGF-C and VEGF-D and is essential for the development of the lymphatic vasculature. Experimental tumors that overexpress VEGFR-3 ligands induce lymphatic vessel sprouting and enlargement and show enhanced metastasis to regional lymph nodes and beyond, whereas a soluble form of VEGFR-3 that blocks receptor signaling inhibits these changes and metastasis. Because VEGFR-3 is also essential for the early blood vessel development in embryos and is up-regulated in tumor angiogenesis, we wanted to determine if an antibody targeting the receptor that interferes with VEGFR-3 ligand binding can inhibit primary tumor growth. Our results show that antibody interference with VEGFR-3 function can inhibit the growth of several human tumor xenografts in immunocompromised mice. Immunohistochemical analysis showed that the blood vessel density of anti-VEGFR-3–treated tumors was significantly decreased and hypoxic and necrotic tumor tissue was increased when compared with tumors treated with control antibody, indicating that blocking of the VEGFR-3 pathway inhibits angiogenesis in these tumors. As expected, the anti-VEGFR-3–treated tumors also lacked lymphatic vessels. These results suggest that the VEGFR-3 pathway contributes to tumor angiogenesis and that effective inhibition of tumor progression may require the inhibition of multiple angiogenic targets. [Cancer Res 2007;67(2):593–9]

Introduction

The vascular endothelial growth factor receptor 3 (*Vegfr3*; *Flt4*) gene is essential for the remodeling and maturation of the embryonic blood capillaries, and, in its absence (*Vegfr3*−/−), embryos die around E10 due to cardiovascular failure (1). After this time point, *Vegfr3* expression in normal embryos becomes restricted to the lymphatic vessels, which develop from the largest embryonic veins (2). In addition to the lymphatic endothelium, fenestrated blood capillaries of some adult organs continue to express low amounts of VEGFR-3 (3). Activation of downstream signaling via the binding of VEGF-C or VEGF-D to VEGFR-3 is required for growth of the lymphatic vasculature (4). The VEGFR-3 receptor homologue in zebrafish embryos is also required for normal blood vessel development (5, 6).

In experimental tumor models, overexpression of VEGFR-3 ligands by tumor cells induced intratumoral and peritumoral lymphangiogenesis and increased metastasis to the regional lymph nodes (7–11), whereas a soluble form of VEGFR-3, which inhibits VEGF-C/VEGF-D signaling, inhibited both lymphangiogenesis and metastasis (9, 12–14). Furthermore, VEGF-C/VEGF-D produced by tumor cells were shown to induce sprouting of lymphatic capillaries and dilation of the draining peritumoral lymphatic vessels (9, 15), thus facilitating lymphatic metastasis, which could be blocked dose-dependently by inhibition of VEGFR-3 signaling using the soluble receptor (16).

Considering that VEGFR-3 is required for hypoxia-driven vascular development and that it interacts with VEGFR-2 (5, 17), and because its expression is up-regulated in angiogenic tumor vessels (18–20), we wished to determine if a monoclonal antibody targeting VEGFR-3 (mF4-31C1), which is capable of preventing ligand binding to the receptor (21), can inhibit primary tumor growth.

Materials and Methods

Mouse, cell lines, and tumors. NCI-H460-LNM35 cells, which are a subline of NCI-H460-N15, a human large-cell carcinoma of the lung (22), and LLC cells were maintained in RPMI 1640 and DMEM, respectively, supplemented with 2 mmol/L L-glutamine, penicillin (100 units/mL), streptomycin (100 μg/mL), and 10% fetal bovine serum (Autogen Bioclear, Calne, United Kingdom). Luciferase (Luc)-tagged sublines of NCI-H460-LNM35 were then established by transfection with AAV-Luc virus, and Luc+ clones were isolated by means of limiting dilution (16, 22). HT-29, SK-RC-29, BxPC-3, HPAC, and PC-3 cells were obtained from the American Type Culture Collection (Manassas, VA). Nude BALB/c (5–6 weeks of age) or severe combined immunodeficient (SCID; 7–9 weeks of age) mice were purchased from Taconic (Cologne, Denmark) or from Charles River Laboratories (Wilmington, MA). Mice were housed under pathogen-free conditions in microisolator cages with laboratory chow and water available *ad libitum*. All experiments and procedures were done in accordance with the Finnish legislation and the U.S. Department of Agriculture, Department of Health and Human Services, and NIH policies on humane care and use of laboratory animals. NCI-H460-LNM35 cells (1×10^7 per SCID mouse and 1.5×10^6 per nude mouse) and LLC cells (1.5×10^6 per mouse) were injected s.c. into the abdomen. All other s.c. xenografts were established by injecting cells (2×10^6 SK-RC-29 or HPAC; 5×10^6 HT-29, PC-3, or BxPC-3) in 50% Matrigel (Collaborative Research Biochemicals, Bedford, MA) s.c. into the flank.

Subcutaneous tumors were allowed to reach a threshold volume (100–500 mm^3) and then the mice were randomized by tumor volume into

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

M. Waltari and T. Holopainen contributed equally to this work.

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Table 1. VEGF-C, VEGF-A, and VEGFR-3 expression by tumor cell lines

Tumor type	Cell line	Ligand concentration			Receptor (VEGFR-3)*
		VEGF-C†	F‡	VEGF-A†	
Epidermoid	A431	500		5,787	1
Breast	DU4475	0		150	1
	MCF-7	0		850	1
	MDA-MB-231	2,200	1/6	3,263	1
	MDA-MB-435	0		3,150	1
	MDA-MB-468	0		15,000	1
	MDA-MB-435 LM2	0		1,180	1
Gastrointestinal	CaCo2	0		250	1
	Colo205	0		4,500	1
	DIFI	0		700	1
	DLD-1	0		700	1
	GEO	0	0/12	4,066	1
	HCT-8	0		2,900	1
	HCT-116	0		2,025	1
	HT-29	0		1,348	1
	LoVo	0		7,000	1
	T84	0		7,137	1
	SW620	0		203	1
	NCI-N87	0		1,443	1
Head and Neck	Cal-27	6,729		5,000	1
	FADU	925	3/3	1,016	1
	Detroit 562	5,881		7,269	1
Leiomyosarcoma	SK-LMS-1	6,286	1/1	6,657	1
Ovarian	OVCAR5	0		3,090	1
	OVCAR8	0	1/4	359	3
	MDAH-2774	2,000		ND	1
	SKOV3	0		481	1
Pancreatic	BxPC-3	0		950	1
	HPAC	0	1/3	2,700	1
	L3.7pL	324		3,800	1
Prostate	Du145	0		550	1
	LNCaP	0	1/3	4,773	1
	PC-3	5,400		2,400	1
Renal	A498	380		1,000	1
	Caki-1	800	3/3	450	1
	SK-RC-29	1,333		30,400	1
NSCLC	A549	1,870		700	1
	NCI-H1650	0		4,500	1
	NCI-H292	0		4,750	1
	NCI-H460	2,278		3,200	1
	NCI-H441	0	2/10	30,666	1
	NCI-H226	0		2,250	1
	Calu-6	0		264	1
	NCI-H358	0		8,409	1
	NCI-H358	0		19,345	1
	NCI-H1975	0		6,493	1
SCLC	NCI-H82	0	0/1		1
Osteosarcoma	KHOS/NP	977	2/2	3,521	1
	MNNG/HOS	2,088		1,570	1
Glioblastoma	U-118 MG	46,875	2/2	26,938	1
	U-87 MG	7,094		16,250	1

Abbreviations: ND, not determined; NSCLC, non–small-cell carcinoma of the lung; SCLC, small-cell carcinoma of the lung.
*Mean fluorescence intensity.
†Concentration in media at 24 h (pg/mL/10^6 cells).
‡Frequency of cell lines expressing VEGF-C in culture.

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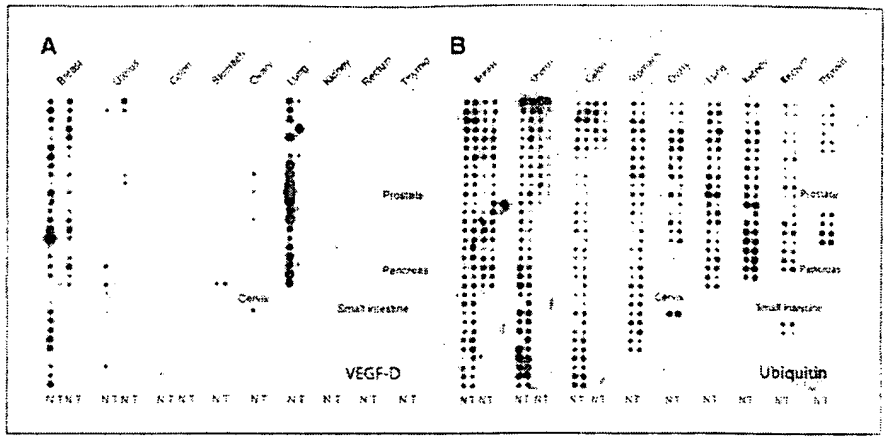

Figure 1. Expression of VEGF-D RNA by human tumors. *A*, to estimate the expression levels of VEGF-D in human tumor samples, we performed a dot blot analysis of VEGF-D mRNA in 241 human tumors (*T*) and corresponding normal tissues (*N*) from individual patients on a cDNA tissue array. Whereas VEGF-D was expressed in many normal tissues, its expression was in general down-regulated in tumors. No difference in the expression of the housekeeping gene *ubiquitin* was detected between the normal and tumor tissues in the same array (*B*).

treatment groups. Tumor-bearing mice were treated with antimouse VEGFR-3 antibody [mF4-31C1 (21), control immunoglobulin G (IgG), bevacizumab (ref. 35; obtained from the Helsinki University Hospital, Helsinki, Finland), or in combination every second day by i.p. or i.v. injection of the antibodies]. Tumor volumes were measured with a caliper and calculated according to the formula V = width × height × depth / 2, derived from the formula for the volume of an ellipsoid (24). Tumor volumes were analyzed using repeated-measures ANOVA with the JMP Statistical discovery package (v. 5.1, SAS Institute, Inc., Cary, NC).

***In vivo* imaging of primary tumors and quantification of bioluminescence signals *in vivo*.** Imaging was done at 1-week time intervals to detect primary tumor growth. For *in vivo* imaging, mice were injected i.p. with D-luciferin (Synchem, Kassel, Germany) at 150 mg/kg of mouse body weight. The light emitted from the bioluminescent tumors was detected with the IVIS Imaging System (Xenogen, Alameda, CA). Images of bioluminescent signals were acquired and the photonic signal intensities were subsequently quantified using the Living Image software (Xenogen).

Antibodies and immunohistology. Blood vessels were visualized by staining tissue sections with monoclonal antibodies against CD31 (rat anti-mouse or Syrian hamster anti-mouse antibodies from PharMingen) or MECA-32 (PharMingen). Goat anti-mouse VEGFR-3 (R&D Systems) was used to stain VEGFR-3 in the tumor sections. A polyclonal rabbit anti-mouse LYVE-1 antibody (25) was used to visualize lymphatic vessels. The primary antibodies were detected with the appropriate Alexa 488 or Alexa

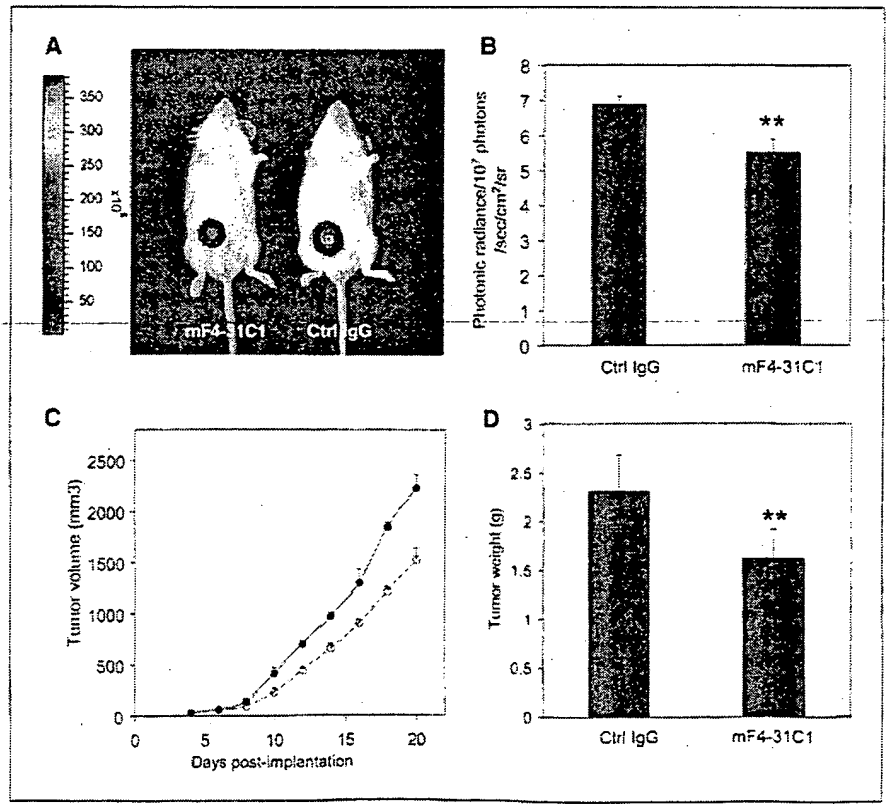

Figure 2. Antibody targeting of VEGFR-3 inhibits the growth of NCI-H460-LNM35 human lung carcinoma. *A*, tumor response to VEGFR-3 inhibition by mF4-31C1 antibodies was assessed by bioluminescent imaging 1 wk after s.c. inoculation of the luciferase tagged tumor cells into SCID mice. In this experiment, the treatment was started simultaneously with tumor cell implantation. Representative bioluminescent images of mF4-31C1- and rat IgG–treated (*Ctrl IgG*) primary tumors with an interpretative color bar. *B*, quantification of the average bioluminescent radiance after mF4-31C1 treatment ($P = 0.0037$; $n = 7$ in mF4-31C1, $n = 11$ in control IgG group). *C*, growth curves of mF4-31C1-treated (*dotted line with open circles*) and control IgG–treated (*line with solid circles*) tumors. *Bars*, SD. *D*, mean primary tumor weights at the end of the treatment; *bars*, SE.
**, $P < 0.01$.

594 (Molecular Probes)–conjugated secondary antibodies or by peroxidase immunohistochemistry using the trichostatin A detection method (Perkin-Elmer, Life Sciences). Whole-mount staining was done on 100-μm-thick frozen sections. The capture ELISA assays were done per manufacturer's instructions (R&D Systems). To assess the VEGF-D expression in different human tumors and corresponding normal tissues from individual patients, we used a tissue array (Cancer Profiling Array I, Clontech) following the manufacturer's instructions.

Necrosis and hypoxia. Hypoxic tumor tissue was detected by i.v. injection of the hypoxia marker pimonidazole hydrochloride (60 mg/kg; Hypoxyprobe kit, Chemicon International; ref. 40), followed by staining of tissue sections with FITC-conjugated monoclonal antibodies (Hypoxyprobe-1 Mab1, Chemicon International) that recognize pimonidazole adducts in hypoxic tissue proteins.

Image analysis. Sections from eight tumors from both treatment groups were viewed under a Leica DM LB microscope, and images were captured with an Olympus DP50 color camera. The samples were viewed at ×25 to ×100 magnifications. For quantitative analysis of the hypoxic and necrotic areas, the Image-Pro Plus software of MediaCybernetics was used. Necrotic areas were quantified from two to four sections per tumor (four tumors per treatment group) and the whole area of a section was used for quantification.

Results

Recent publications have suggested that VEGFR-3 is expressed in a large fraction of human cancers (27–30). Although we originally cloned VEGFR-3 from the HEL erythroleukemia cell line, our studies had indicated that this receptor is rarely expressed by nonvascular tumor cells in monolayer culture (23, 31, 32). We therefore carried out flow cytometric analysis on 52 tumor cell lines, of which only two (OVCAR8 and HEL) were positive for VEGFR-3 (Table 1). The VEGFR-3-specific staining with the hF4-3C5 antibody, which recognizes human VEGFR-3 (33), in the OVCAR8 or HEL cell lines was inhibited by incubating the cells with the corresponding immunogen (data not shown). Many of the tumor cell lines secreted VEGFR-3 ligand VEGF-C (18 of 52, 35%; Table 1), whereas none of the cell lines tested secreted VEGF-D as judged by a capture ELISA. All tumor cell lines secreted VEGF165 (detection limits in the capture ELISA analysis were 62 pg/mL for VEGF-D, 94 pg/mL for VEGF-C, and 15 pg/mL for VEGF165; Table 1). Analysis of RNA from 241 human tumors and corresponding normal tissues from individual patients indicated that many normal tissues expressed VEGF-D mRNA, with breast and lung tissues displaying notably high expression. Total VEGF-D mRNA was in general down-regulated in tumors (Fig. 1). It should be noted, however, that VEGF-D expression has been reported in several human tumor types and in tumor stromal cells, such as inflammatory cells (34). Indeed, the analysis of total RNA would not be sensitive enough to high regional expression of VEGF-D.

To determine if the inhibition of VEGFR-3 signaling by a blocking antibody against the receptor would have an effect on primary tumor growth, we treated tumor-bearing nude or SCID mice with the mF4-31C1 antibody. This antibody inhibits ligand binding and phosphorylation of murine VEGFR-3 and blocks VEGF-C-stimulated downstream signaling (21, 33).[4] We first studied the effect of mF4-31C1 in early phases of tumor growth by using luciferase-expressing NCI-H460-LNM35 cells in SCID mice (16, 22). Inhibition of tumor growth was evident already after four injections of mF4-31C1 antibodies (Fig. 2*A* and *B*). We then investigated later stages of primary tumor growth and expanded our study to other types of tumors. Our results showed that mF4-31C1 antibodies inhibited the s.c. growth of several different tumor cell lines in nude mice (Fig. 3). Variable inhibition of primary tumor growth by 30% to 44% occurred in xenograft models of human pancreatic carcinomas (BxPC-3 and HPAC), a renal carcinoma (SK-RC-29), a colon carcinoma (HT-29), a prostate carcinoma (PC-3), a large-cell lung carcinoma (NCI-H460-LNM35), and in a mouse lung carcinoma (LLC). The only tumor model tested, which seemed to be very resistant to the anti-VEGFR-3 treatment, was an aggressive, subclone of the human colon carcinoma SW480 (ref. 22; Supplementary Table S1).[5]

Figures 2*C* and 3 show examples of the growth curves of NCI-H460-LNM35, HT-29, BxPC-3, and PC-3 tumors treated with i.v. (Fig. 2*C*) or i.p. (Fig. 3) injections of the mF4-31C1 antibodies (*open circles, dotted line*) or with various controls (*solid circles*). Significant inhibition of tumor growth was obtained in 7 of 11 experiments with a dose of 40 mg/kg of mF4-31C1 (*P* values are shown in Supplementary Table S1). Half of this dose (20 mg/kg) was enough to significantly inhibit tumor growth in the NCI-H460-LNM35 xenograft model when administered into the tail vein (Fig. 2*C* and *D*). Although a lower dose (10 mg/kg; Fig. 3*B* and *C*, *solid boxes*) seemed to decrease tumor growth, this effect did not reach statistical significance.

Histologic analysis using antibodies against blood vascular endothelial markers MECA-32 or CD31 revealed that the mF4-31C1–treated tumors had significantly decreased blood vessel density in comparison with the control antibody–treated tumors (Fig. 4*A*, *B*, *E*, and *F*; $P \leq 0.001$), suggesting that the antitumor effect was through inhibition of tumor angiogenesis. Reduction in blood vessel density was observed both in the tumor periphery and in the core of the treated tumors (Fig. 4*F*). These results suggested that VEGFR-3 was expressed in the tumor blood vessels. To confirm this, we double stained tumor sections with antibodies against VEGFR-3 and vascular marker proteins CD31 and LYVE-1. Many of the blood vessel endothelial cells in the NCI-H460-LNM35 tumors were indeed positive for VEGFR-3 in frozen sections double stained for CD31 (Fig. 5*A*), whereas they lacked staining for the lymphatic marker protein LYVE-1 (data not shown). In addition, the mF4-31C1–treated tumors lacked intratumoral lymphatic vessels (Fig. 4*C* and *D*), whereas no effect was detected in the preexisting lymphatic vessels in the skin overlying the tumor (Fig. 4*C* and *D*, *white arrows*), in accordance with previous results (21).

The effect of mF4-31C1 antibody was more pronounced than the mere tumor volume suggested because the mF4-31C1–treated tumors showed significantly more necrosis than the control antibody–treated tumors (Fig. 5*B–D*). This suggests that anti-VEGFR-3 therapy led to tumor tissue necrosis. In several cases, only a thin peripheral zone of viable tumor remained adjacent to the border of the tumor mass and surrounding host tissues in the mF4-31C1–treated tumors. The hypoxia marker pimonidazole hydrochloride stained the rim of hypoxic cells between the necrotic area and the viable tumor mass in both mF4-31C1– and control antibody–treated tumors (Fig. 5*C* and *D*, *arrows*). In addition to that, the hypoxia marker stained tumor cells inside the tumor mass in the mF4-31C1–treated tumors (Fig. 5*D*, *arrowheads*), indicating the presence of hypoxia in additional areas. Furthermore, a reduced

[4] Unpublished observations.

[5] Dr. Tatiana Petrova, personal communication.

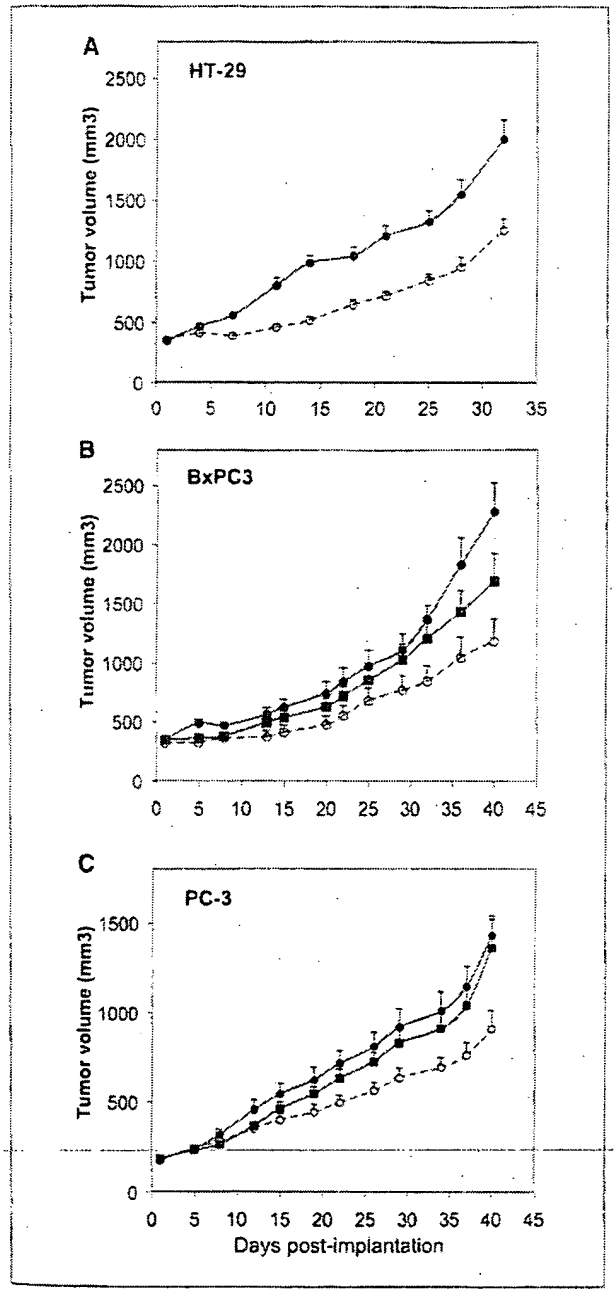

Figure 3. Anti-VEGFR-3 treatment inhibits primary tumor growth in several tumor models. Tumor cells were injected s.c. into nude mice. Tumor volumes were measured by using a caliper. Antibody treatment was started when the tumors were palpable. *Points*, mean tumor volumes during the treatment; *bars*, SE. *Line with solid circles*, control treatment (PBS); *dotted line with open circles*, mF4-31C1, 40 mg/kg; *line with solid boxes*, mF4-31C1, 10 mg/kg.

blood vessel density correlated with increased hypoxia around the vessels in the tumor mass (Fig. 5*E* and *F*).

Bevacizumab is a humanized monoclonal antibody against human VEGF, which has been approved for clinical use against metastatic colorectal cancer (35). Bevacizumab was very effective in inhibiting the growth of the NCI-H460-LNM35 xenografts at 5 mg/kg (~70% inhibition; $P = 0.002$). When a suboptimal dose (1.5 mg/kg) was used instead, bevacizumab inhibited primary tumor growth to a similar extent as the mF4-31C1 antibodies (35% and 45% inhibition, respectively), whereas a combination of both of these antibodies gave 62% inhibition of primary tumor growth (Supplementary Fig. S1). However, this difference was not statistically significant.

Discussion

Previous studies have shown that blocking the binding of VEGF-C and VEGF-D to VEGFR-3 using the soluble VEGFR-3-IgG Fc fusion protein inhibits tumor lymphangiogenesis and tumor metastasis to lymph nodes (9, 12–14, 16). We show here that treatment of tumor-bearing mice with the mF4-31C1 monoclonal antibody, which antagonizes ligand binding to VEGFR-3, inhibits primary tumor growth in several different experimental tumor models. Surprisingly, the efficacy of anti-VEGFR-3 treatment did not correlate with VEGF-C/VEGF-D expression by the corresponding

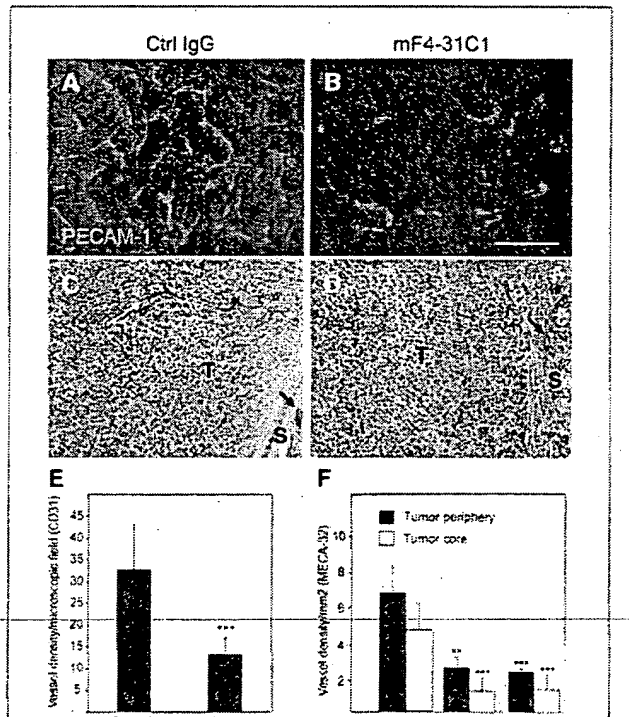

Figure 4. Decreased blood vessel density in anti-VEGFR-3–treated tumors. At the end of the treatment, the tumors were excised and prepared for histologic analysis. Blood vessels were stained with an anti-PECAM-1 antibody (*A* and *B*, *green*). Anti-VEGFR-3–treated tumors (*B*) showed a decreased density of PECAM-1–positive vessels when compared with control antibody–treated tumors (*A*). Staining with anti-LYVE-1 antibodies revealed that the mF4-31C1-treated tumors lacked intratumoral and peritumoral lymphatic vessels (*D*) whereas control antibody–treated tumors contained LYVE-1–positive vessels (*C*, *white arrow*). *C* and *D*, *black arrows*, normal lymphatic vessels in the skin overlying the tumor. *E*, quantification of PECAM-1–positive vessels in the treated NCI-H460-LNM35 tumor xenografts. *F*, quantification of MECA-32–positive vessels in the periphery and core of the treated SK-RC-29 tumor xenografts. Vessels were counted from three representative sections of each tumor (five per group). DC101, antimouse VEGFR-2 antibody. *Bars*, SD. *T*, tumor; *S*, skin. Magnification, ×200 (*A* and *B*); ×100 (*C* and *D*). Whole-mount staining of 100-μm sections (*A* and *B*) or staining of 7-μm sections on glass slides (*C* and *D*). **, $P < 0.01$; ***, $P < 0.001$. Bar, 50 μm.

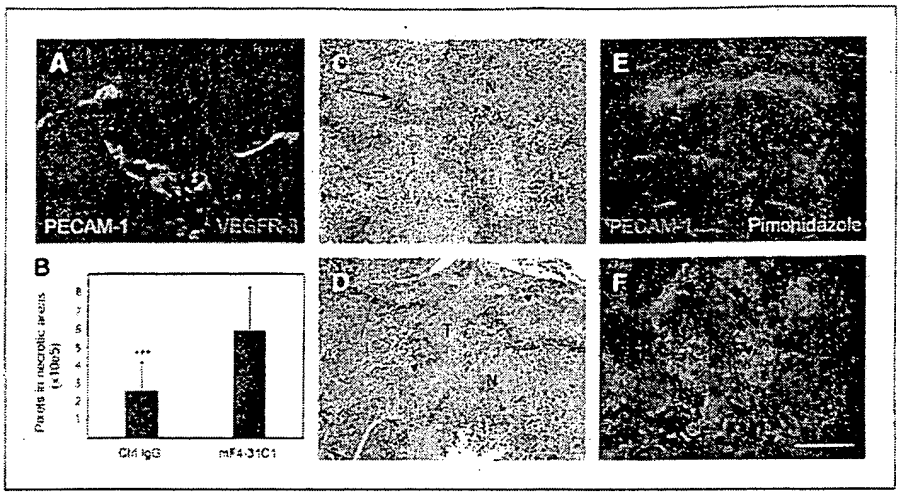

Figure 5. Anti-VEGFR-3–treated tumors show increased necrosis. *A*, sections from NCI-H460-LNM35 tumors were stained for VEGFR-3 (*red*). Blood vessels were visualized by anti-PECAM-1 staining (*green*). *B*, quantification of necrotic areas in mF4-31C1– and control antibody–treated tumors. *Bars*, SD. *C* and *D*, tumors were excised and hypoxic areas were visualized with a monoclonal antibody that recognizes pimonidazole adducts in hypoxic tissue (*C* and *D*, *brown*). *Arrows*, rim of hypoxic cells between the necrotic areas (*N*; *blue staining*) and viable tumor mass. Note that in the mF431C1-treated tumors, tumor cells inside the tumor mass stain brown for the hypoxia marker (*D*, *black arrows*). *E* and *F*, thick (100 μm) tumor sections were stained with antibodies against PECAM-1 (*red*) and pimonidazole adducts (*green*). The mF4-31C1–treated tumors (*F*) contained fewer blood vessels but larger hypoxic areas when compared with control antibody–treated tumors (*E*). *T*, viable tumor tissue. Magnification ×200 (*A*), ×100 (*C–F*). ***, $P < 0.001$. Bar, 50 μm.

tumor cell lines *in vitro* (Table 1). This may reflect the fact that VEGF-C/VEGF-D–negative tumor cell lines can induce ligand expression *in vivo* (13) and that inflammatory cells *in vivo* contribute significantly to VEGF-C/VEGF-D production (36). The inhibitory effect seemed to be through inhibition of angiogenesis because the anti-VEGFR-3–treated tumors contained significantly less blood vessels than the control antibody–treated tumors.

In a previous study, the rat anti-VEGFR-3 monoclonal antibody AFL4 was reported to inhibit tumor xenograft growth in mice by compromising the integrity of tumor blood vessels, leading to microhemorrhages (20). However, in our hands, the AFL4 antibody does not inhibit ligand binding or phosphorylation of VEGFR-3 nor does it block VEGF-C–stimulated cell proliferation (21). This means that the reported *in vivo* effects of the AFL4 are mediated by a mechanism other than the direct inhibition of ligand binding as with mF4-31C1 (21). For example, the AFL4 antibody could inhibit receptor dimerization or promote receptor internalization or antibody-dependent cytotoxicity. In our present study, we started the treatment when palpable, established tumors were detected in the mice, whereas in the study by Kubo et al. (20), the treatment was started simultaneously with tumor implantation and no significant inhibition was observed when treatment was started later. After the first submission of our manuscript, Roberts et al. (37) reported on decreased tumor growth in an orthotopic VEGF-C expression vector–transfected human mammary carcinoma xenograft in nude mice that were treated with the mF4-31C1 antibody for 6 weeks starting at the time of tumor cell inoculation. Their treated and untreated tumors were less than 100 and 150 mm^3 in size, respectively. This would correspond to a spheroid tumor diameter of 5.8 and 6.6 mm, respectively. Because such size range makes quantitative assessment very difficult in our experiments, we started the antibody treatment only after the tumors reached this size. Besides inhibiting tumor angiogenesis, the mF4-31C1 treatment blocked formation of tumor-associated lymphatic vessels that have been associated with lymphatic metastasis, whereas it did not affect preexisting lymphatic or blood vessels. Similarly, no effect on mature lymphatic vessels or blood vasculature was observed when the VEGFR-3 pathway was blocked by the soluble receptor produced transiently by adenovirus or adeno-associated virus (14, 26). Further work should define the mechanisms involved in the reduced tumor angiogenesis up on VEGFR-3 blockade.

Tumor angiogenesis is promoted by multiple pathways that include both oncogene-driven and tumor-associated, hypoxia-driven expression of proangiogenic factors such as VEGF and perhaps down-regulation of angiogenic suppressors (38). Tumor cells and stromal macrophages in several tumor types express VEGF-C or VEGF-D, and their receptor VEGFR-3 is commonly expressed in the angiogenic tumor vasculature and stromal macrophages (36), but not, in general, in tumor cells or in most normal vessels (39). Our results in this study suggest that VEGF-C/VEGF-D and their receptors contribute to angiogenesis and growth in at least some tumors. Monotherapy with a single antiangiogenic agent may not be sufficient to counteract the numerous angiogenic factors produced by cancer cells and/or stromal cells during tumor progression. The usefulness of VEGFR-3 blocking antibodies will next be further explored in combination studies with other antiangiogenic and antitumor agents. Indeed, in some tumor types, effective inhibition of tumor progression may require inhibition of multiple angiogenic targets.

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Involvement of vascular endothelial growth factor receptor-3 in maintenance of integrity of endothelial cell lining during tumor angiogenesis

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Vascular endothelial growth factor (VEGF) plays a major role in tumor angiogenesis. VEGF-C, however, is thought to stimulate the growth of lymphatic vessels because an expression of its specific receptor, VEGF receptor-3 (VEGFR-3), was demonstrated to be restricted to lymphatic vessels. Here we demonstrate that the inactivation of VEGFR-3 by a novel blocking monoclonal antibody (mAb) suppresses tumor growth by inhibiting the neo-angiogenesis of tumor-bearing tissues. Although VEGFR-3 is not expressed in adult blood vessels, it is induced in vascular endothelial cells of the tumor-bearing tissues. Hence, VEGFR-3 is another receptor tyrosine kinase involved in tumor-induced angiogenesis. Micro-hemorrhage in the tumor-bearing tissue was the most conspicuous histologic finding specific to AFL4 mAb-treated mice. Scanning microscopy demonstrated disruptions of the endothelial lining of the postcapillary venule, probably the cause of micro-hemorrhage and the subsequent collapse of the proximal vessels. These findings suggest the involvement of VEGFR-3 in maintaining the integrity of the endothelial lining during angiogenesis. Moreover, our results suggest that the VEGF-C/VEGFR-3 pathway may serve another candidate target for cancer therapy. (Blood. 2000;96:546-553)

Introduction

Angiogenesis is essential for tumor progression because it allows oxygenation and nutrient perfusion of the tumor. In the absence of neovascularization, a solid tumor cannot form a large mass.[1-3] Angiogenesis is a complex multistep process by which blood vessels are formed from the preexisting vasculature. Previous studies of null mutant mice have demonstrated that development of the embryonic vascular system requires the coordinated expression of various receptor tyrosine kinases (RTKs) and their ligands.[4] Among these ligands, vascular endothelial growth factor (VEGF) has been shown to play a major role throughout angiogenesis. VEGF action is mediated by 2 RTKs, VEGFR-1 (FLT-1) and VEGFR-2 (FLK-1/KDR), expressed primarily by endothelial cells (ECs). VEGF regulates multiple EC activities such as proliferation, migration, tube formation, and permeability.[5-8] Some molecules, such as angiopoietins (Ang1 and Ang2)/Tie2, are implicated in the later stages of vascular development—ie, during vascular remodeling and maturation.[9-11] Based on studies using reagents that neutralize each ligand, it has been suggested that those RTKs involved in the embryonic process are also involved in tumor angiogenesis.[12-16]

Recently, VEGFR-3 was identified as an endothelial-specific RTK related to VEGF receptors.[17] VEGFR-3 is induced in all endothelial cells during early embryogenesis, though its expression eventually disappears from the vascular ECs of adult tissues.[18] In contrast to its transient expression in vascular ECs, VEGFR-3 is expressed constitutively by the adult lymphatic endothelium.[19] VEGF-C, a new member of the platelet-derived growth factor (PDGF)/VEGF family, was first identified as a ligand for VEGFR-3.[20] Among several distinct forms of VEGF-C generated by stepwise proteolysis, the maturely processed VEGF-C could also activate VEGFR-2.[21] Moreover, another ligand, named VEGF-D[22] for VEGFR-3, has been identified recently, illustrating the complex ligand–receptor relationship, which poses a problem for understanding the role of VEGFR-3 during vasculogenesis.

Transgenic overexpression of VEGF-C in the skin has been shown to induce hyperplasia of the lymphatic vasculature (lymphangiogenesis), leaving the vascular structure unaffected. This observation implies a lymphatic-specific role for VEGF-C/VEGFR-3.[23,24] Another report using the cornea assay,[25] however, demonstrated that VEGF-C could induce angiogenesis of adult tissues. Mice bearing a null mutation of the VEGFR-3 gene display defects in vascular remodeling, indicating a role for VEGFR-3 in angiogenesis.[26] Nonetheless, because VEGFR-3 is expressed by vascular ECs in the embryo but not the adult, the role of VEGFR-3 in adult mice is yet to be determined. We generated an antagonistic monoclonal antibody (mAb) against VEGFR-3 to elucidate the role of VEGFR-3 in tumor angiogenesis. We show that VEGFR-3 is indeed involved in tumor angiogenesis and is essential for maintaining the integrity of the endothelial sheet.

Materials and methods

Mice

Six- to 8-week-old nude (nu/nu) mice were purchased from SLC (Shizuoka, Japan). VEGFR-3 null mutant embryos[26] were maintained and mated in our animal facility.

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Cell culture

The C6 rat glioblastoma cell line (a gift from Dr H. Kataoka, Kyoto University, Kyoto, Japan), the F-2 murine endothelial cell line[27] (a gift from Dr K-I Toda, Kyoto University, Kyoto, Japan), and the 293 human embryonic kidney cell line (American Type Culture Collection, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO/BRL, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) (HyClone Laboratories, Logan, UT). PC-3 human prostate adenocarcinoma cell line (a gift from Dr N. Itoh, Kyoto University, Kyoto, Japan) were maintained in RPMI 1640 medium (GIBCO/BRL) containing 10% FCS.

Gene transfection

For the preparation of Fc chimeric proteins, cells from the 293 cell line were plated on a 100-mm tissue culture dish to reach 70% confluence the next day and were transfected with 15 μg pCDM8-VEGFR-3-Fc (described below), pCDM8-VEGFR-1-Fc,[28] or pCDM8-VEGFR-2-Fc[29] plasmid DNA mixed with 25 μg Trans-IT (Mirus, Madison, WI) according to the manufacturer's instructions. Transfected cells were grown in DMEM/F12 medium, and culture supernatants were harvested 5 days after transfection. Fusion protein was purified using protein G–Sepharose columns (Pharmacia Biotech, Uppsala, Sweden).

Plasmid vector containing murine VEGFR-3 cDNA (P2B1S neo/mFLT4[17]) was kindly provided by Dr W. I. Wood (Genentech, Cambridge, MA). cDNA containing full-length VEGFR-3 with *Not* I and *Sal* I restriction sites at the 5′ and 3′ ends, respectively, was obtained from P2R1S neo/mFLT4 and subcloned into the expression vector pCDNA3.

Generation of anti-VEGFR-3 monoclonal antibody

A 2.3-kb fragment of murine VEGFR-3 cDNA (positions 45-2354 in the GeneBank L07296), encoding the extracellular domain of VEGFR-3, was subcloned into the expression vector pCDM8-hIgG.[29] Rat monoclonal antibodies against VEGFR-3-Fc protein were produced using standard methods as described.[29] In brief, an 8-week-old Wistar rat was first immunized subcutaneously with 500 μg VEGFR-3-Fc protein in complete Freund's adjuvant (Difco, Detroit, MI) and then was administered 3 intraperitoneal shots of 250 μg VEGFR-3-Fc protein in Freund's incomplete adjuvant (Difco) in alternating weeks and finally was given an intravenous boost of 100 μg VEGFR-3-Fc protein. Three days after the boost, the spleen cells were harvested and fused with the murine myeloma X63Ag8. Undiluted supernatants from hybridoma were screened by enzyme-linked immunosorbent assay (ELISA) plates coated with 50 ng/mL VEGFR-3-Fc; VEGFR-2-Fc[29] and VEGFR-1-Fc were used as controls.[28] Positive hybridomas were cloned by the limiting dilution technique and were subcloned twice.

VEGF-C/VEGFR-3 binding inhibition assay by ELISA

The N-terminal signal sequence of mouse stem cell factor (MMU44725; 198-279) and the 5 repeated *myc*-tag sequences were inserted to a 5′ end of the cDNA fragment corresponding to the mature VEGF-C (U43142; 705-1052),[21] which was generated by reverse transcription–polymerase chain reaction amplification of mRNA prepared from PC-3 cells (5-myc-VEGF-C). For the binding inhibition study, ELISA plates coated with 50 ng/mL VEGFR-3-Fc protein were first incubated with various dilutions of mAbs and then with the conditioned medium (CM) of 293 cells transfected by the 5-myc-VEGF-C gene. Binding with 5-myc-VEGF-C was detected by the anti-myc tag antibody (9E10) (Santa Cruz Biotechnology, Santa Cruz, CA) and then by horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Zymed, San Francisco, CA). Plate-bound enzymic activity was detected by using 3′,3′,5′,5′-tetramethylbenzidine (Chemo-Sero Therapeutic Research Institute, Kumamoto, Japan), and absorbance of each well was measured using the ELISA reader.

Tumor transplantation

Six- to 8-week-old nude (nu/nu) mice (SLC, Shizuoka, Japan) underwent subcutaneous transplantation of 2×10^6 C6 rat glioblastoma cells or PC-3 prostate cancer cells in 0.1 mL phosphate-buffered saline (PBS) on the right flank. Subcutaneous injections of mAbs were given on the left flank of mice. Tumor size was measured in 2 dimensions, and the volume was calculated using the formula, width^2 × length/2. After 14 days, all mice were humanely killed and autopsied.

Immunohistochemistry

Tissues were fixed in 4% paraformaldehyde in PBS overnight, embedded in paraffin and sectioned at 5 to 7 μm. The sectioned specimens were incubated first in bleaching solution (methanol, 0.2% NaN_3; 0.6% H_2O_2) for 30 minutes at room temperature to block endogenous peroxidase. After rehydration, the sections were blocked by incubation with 1% bovine serum albumin in PBS–0.1% Tween 20 (PBS-T) for 20 minutes at room temperature and then incubated overnight with respective primary antibodies: rat anti-VEGFR-3 mAb, AFL4 (20 μg/mL); rat anti-VEGFR-2 mAb, AVAS12[29] (10 μg/mL); and rat mAb for murine PECAM-1 (5 μg/mL, Mec13.3; PharMingen, San Diego, CA). After they were washed 3 times in PBS-T for 10 minutes each at room temperature, the sections were incubated with 1 μg/mL HRP-conjugated anti-rat IgG(H+L) (Biosource, Camarillo, CA) for 1 hour at room temperature. After washing with 3 exchanges of PBS-T, the enzymatic reaction with enhanced DAB substrate kit (TSA–Indirect; NEN Life Science Products, Boston, MA) was allowed to proceed until the desired color intensity was reached. For immunohistochemical analysis of AFL4-treated mice, tissues were incubated with a biotinylated anti-PECAM-1 antibody (1/100) as a primary antibody, and HRP-conjugated streptavidin (Zymed) (1/1000) was used as a developing reagent.

The densities of PECAM-1$^+$ and VEGFR-3$^+$ vessels were calculated according to the method described by Gasparini and Harris.[30] A minimum of 5 fields (×200) was counted per slide.

Whole-mount immunostaining was performed according to the protocol previously described.[31] In some experiments, stained whole-mount specimens were embedded in polyester wax (BDH, Poole, UK) and sectioned.

Scanning electron microscopy (SEM)

Tumor masses with surrounding tissues were dissected carefully and fixed with 3% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4). The method of SEM observation was as previously reported.[32] In brief, the specimens were postfixed with OsO_4 and were hydrolyzed with 8N HCl for 25-60 minutes at 60°C. After a brief rinse, the specimens were dehydrated through a graded series of ethanol, immersed in isoamyl acetate, critical-point dried, coated with platinum, and observed with an SEM (Hitachi S-800, Tokyo, Japan).

Western blot analysis

Western blot analysis was performed as described.[29] The filter was probed with 2 μg/mL anti-VEGFR-3 mAb or a control mAb, followed by treatment with HRP-coupled goat anti-rat IgG, and visualized using the enhanced chemiluminescence reagent (NEN Life Science Products).

Phosphorylation assay

F2 cells were grown to subconfluence in DMEM supplemented with 10% FCS. The medium was removed and replaced with fresh DMEM containing 10% FCS with or without antibodies (AFL4, rat IgG fraction 50 μg/mL) for 15 minutes; this was followed by stimulation with one-fifth diluted VEGF-C CM. Fifteen minutes after VEGF-C stimulation, the cells were lysed in lysis buffer (10 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% NP-40, 0.2 mmol/L PMSF, 0.5 mmol/L sodium vanadate, 2 mmol/L sodium fluoride). VEGFR-3 was immunoprecipitated from cell lysates by protein G–Sepharose after incubation with the anti-VEGFR-3 mAb. Proteins were resolved with sodium dodecyl sulfate–polyacrylamide gel electrophoresis under nonreducing conditions and were probed with HRP-conjugated anti-phosphotyrosine mAb (PY20; Transduction Laboratories, Lexington, KY). Filters were reprobed with anti-VEGFR-3 mAb to measure the amount of protein loaded on each lane.

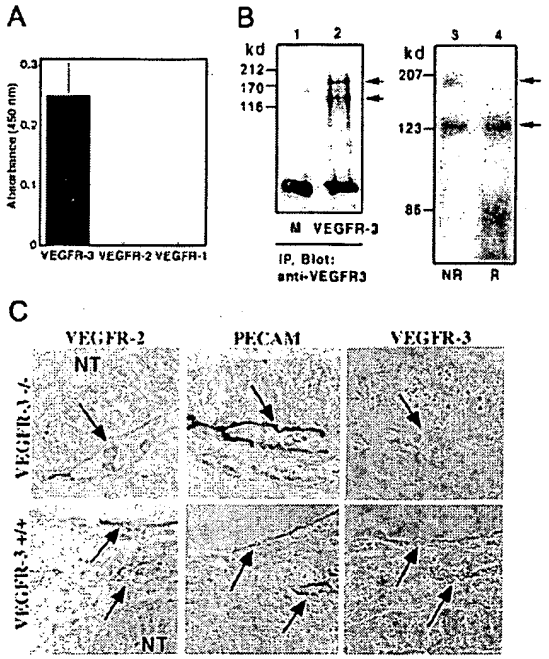

Figure 1. Specificity of AFL4. (A) AFL-4 binding to VEGFR-1-Fc, VEGFR-2-Fc, or VEGFR-3-Fc was analyzed by ELISA. The absorbance at 450 nm is depicted. Each bar represents the mean ± SEM of triplicate assays. (B) Cell lysates from the 293 cell line transiently transfected with murine VEGFR-3 DNA (lane 2) or mock transfected (lane 1, M) were precipitated with AFL4 and immunoblotted with the same antibody. (IP, immunoprecipitation.) Two bands of 195- and 125-kd proteins were detected in lane 2 under reducing conditions. Through immunoblotting of the cell lysates from the murine EC line F2 with anti-VEGFR-3 mAb, 2 bands were detected in nonreducing conditions (NR), whereas only a 125-kd band was seen under reducing conditions (R) (lanes 3, 4). Arrows denote the positions of the unprocessed 195-kd form and the proteolytically processed 125-kd form of VEGFR-3. (C) Specificity of AFL4 in tissue sections. Sagittal sections were prepared from embryonic day 9.5 VEGFR-3$^{-/-}$ or VEGFR-3$^{+/+}$ embryos and stained with AFL4, anti-PECAM-1 mAb, and anti-VEGFR-2 mAb. Arrows indicate blood vessels in the mesenchymal region around the neural tube (NT). Note that all PECAM-1$^+$ cells are also VEGFR-2$^+$ and VEGFR-3$^+$ at this stage of embryonic development.

Results

Production of an antagonistic anti-mouse VEGFR-3 mAb

After screening more than 500 clones, 1 clone, AFL4 (IgG2ak), was isolated as reacting specifically to VEGFR-3-Fc but not to VEGFR-1-Fc or VEGFR-2-Fc (Figure 1A). Two polypeptides were precipitated and immunodetected with AFL4 in cell lysates of VEGFR-3 gene-transfected 293 cells but not from mock-transfected cells (Figure 1B; lanes 1, 2). Through immunoblot analysis of total extracts of the F-2 endothelial cell line,[27] 2 bands of 195- and 125-kd were detected with AFL4 (Figure 1B; lane 3) under nonreducing condition, whereas only the 125-kd band was observed under reducing conditions (Figure 1B; lane 4). These results are consistent with the previous observation that a 175-kd precursor of VEGFR-3 matures to a 195-kd form, which is then proteolytically cleaved into the 125-kd and 75-kd fragments,[33] each linked by disulfide bonds.

To further evaluate the specificity of AFL4, serial transverse sections were prepared from E9.5 VEGFR-3$^{-/-}$ or VEGFR-3$^{+/+}$ embryos and immunostained by AFL4. The AFL4 immunostaining was seen in the vascular endothelial lining of the wild-type embryo but not in that of the VEGFR-3$^{-/-}$ embryo, whereas the expression of VEGFR-2 and PECAM-1 was detected in both groups (Figure 1C). From these results, we concluded that AFL4 is specific to VEGFR-3 and can be used for various purposes, including immunoprecipitation, immunoblotting, and immunostaining of fixed tissues.

We next investigated whether AFL4 blocks the function of VEGFR-3. AFL4 could block the binding of myc-tagged VEGF-C to ELISA plates coated with VEGFR-3-Fc, whereas anti-VEGFR-2 mAb (AVAS12)[29] could not, indicating that AFL4 recognizes the ligand-binding site of VEGFR-3 (Figure 2A). Although these analyses did not permit a precise determination of the binding affinity of AFL4 for VEGFR-3, the IC_{50} for AFL4 inhibition of VEGF-C binding to VEGFR-3 was estimated to be 0.5 μg/mL. We also examined whether the blocking of ligand binding leads to the suppression of receptor signaling. We stimulated F2 cells by VEGF-C in the presence of AFL4 or AVAS12. Tyrosine phosphorylation of immunoprecipitated VEGFR-3 was measured by anti-phosphotyrosine mAb. Compared with the control, AFL-4 treatment resulted in 6- and 3-fold reduction of VEGF-C–induced tyrosine phosphorylation at 125-kd and 195-kd bands, respectively (Figure 2B).

Lymphatic specific expression of VEGFR-3 protein in adult mouse

Although a previous study[18] and Figure 2 demonstrated VEGFR-3 expression in the vascular EC of early embryos, AFL4 recognized only lymphatic vessels in later life. Figure 3 showed the whole-mount immunostaining of the mesentery of E17 embryos, in which lymphatic vessels easily could be distinguished morphologically from vein and artery running in parallel (Figure 3A-C). In cross-sections, staining could be found only in the endothelial cells of the lymphatic vessels that did not contain blood cells in the lumen (Figure 3D).

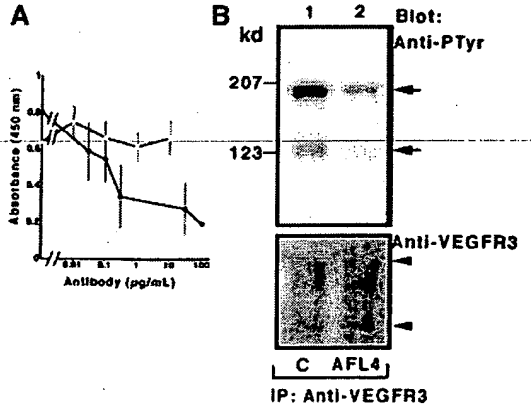

Figure 2. AFL4 blocks VEGFR-3 function by inhibiting VEGF-C binding. (A) Inhibition of binding of VEGFR-3 to VEGF-C by AFL4. Culture supernatant of 293 cells transfected with 5 myc-tagged VEGF-C DNA was incubated with various doses of AFL4 (●) or anti-VEGFR-2 mAb (○) and added to microtiter plates coated with mVEGFR-3-Fc. Binding was quantified by using the anti-myc mAb as a primary antibody, and absorbance at 450 nm was determined. Data indicate the background-corrected mean ± SEM from triplicate wells. (B) Tyrosine phosphorylation of VEGFR-3 in F2 cells was induced by VEGF-C CM in the presence of control IgG (lanes 1, 3) or AFL4 (lanes 2, 4). Total cell lysates were immunoprecipitated with anti-VEGFR-3 mAb and subjected to serial immunoblotting with anti-phosphotyrosine antibody (upper) and anti-VEGFR-3 mAb (lower). Arrows and arrowheads denote the positions of 195-kd and 125-kd forms of VEGFR-3. Relative density of bands against 125-kd bands of the control lanes (lane 1, upper and lower panels) were 195-kd/125-kd 3.7/1 (lane 1, upper), 0.9/0.2 (lane 2, upper), 0.78/1 (lane 1, lower), and 0.6/1.1 (lane 2, lower). Reduction ratios after correction by the amount of protein were 0.32 and 0.18 for 195-kd and 125-kd bands, respectively.

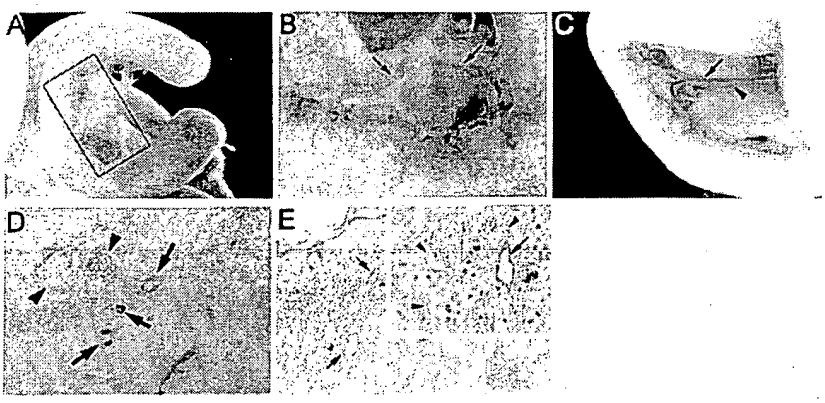

Figure 3. Lymphatic-specific expression of VEGFR-3 in embryonic day 17 (E17) embryos and adult dermis. (A) Whole-mount staining of the mesentery of E17 embryos by AFL4. A fraction of vascular system is stained. (B) Higher magnification of the marked area in panel A demonstrates that lymphatic vessels with typical sac-like structure are stained (arrows). (C) PECAM-1 staining of the same region shows that blood vessels (arrow) and lymphatic vessels (arrowhead) are stained. (D) A section of the E17 mesentery illustrating stained endothelial vessel (arrow) and an unstained blood vessel (arrowhead) (×200). Note that all stained vessels do not contain hematopoietic cells. (E) A section of an adult skin illustrates VEGFR-3$^+$ (arrows) and VEGFR-3$^-$ vessels (×100). Higher magnification (inset, ×200) reveals that VEGFR-3$^+$ vessels (arrows) do not contain hematopoietic cells, whereas unstained blood vessels do (arrowheads).

We next examined VEGFR-3 expression in adult cutaneous tissues. Cells lining the luminal wall of a vascular structure (indicated by arrows) were immunostained by AFL4 (Figure 3E). In contrast to the lack of reactivity to blood vessels containing hematopoietic cells (indicated by arrowheads), all AFL4-reactive vessels did not contain blood cells, suggesting lymphatic specific expression of VEGFR-3. This staining pattern corroborates well with previous in situ analysis showing that VEGFR-3 is specific to ECs lining the lymphatic vessels.[18]

To determine the role of VEGFR-3 in the maintenance of the adult lymphatic system, 1 mg AFL4 was injected subcutaneously into 8-week-old mice on alternating days for up to 3 weeks. During this 3-week period of continuous AFL4-injection, we could not detect any gross abnormality in the treated mice compared with the control mice, which were treated with non-antagonistic anti-VEGFR-2 mAb or PBS (data not shown). Thus, VEGFR-3 appeared not to be essential for the maintenance of the adult lymphatic system.

AFL4 suppresses the growth of xenogenic tumors in nude mice

The antagonistic anti-VEGFR-3 mAb enabled us to examine the involvement of VEGFR-3 in tumor-induced neo-angiogenesis. For this purpose, we first used the C6 glioblastoma cell line, which grows aggressively in the nude mouse.[33] C6 cell line has been shown to secrete VEGF[34] and VEGF-C.[35]

To determine the effect of AFL4 treatment on the C6 growth, 200, 600, or 1000 μg purified AFL4 was injected on alternating days for 12 days into mice grafted with 2×10^6 C6 tumor cells. As controls, 600 μg AVAS12, nonantagonistic anti-VEGFR-2 mAb was injected in the same manner. The size of tumor was measured from day 5 to day 14 after the tumor transplantation. As shown in Figure 4A and Table 1, AFL4 treatment inhibited tumor growth at all doses. Because 200 μg showed less effect than other doses, we decided to use 600 μg for subsequent experiments.

To determine the timing of VEGFR-3 involvement in tumor progression, 3 protocols for antibody injection were tested: (1) day 0 to day 12, (2) day 0 to day 6, and (3) day 7 to day 13. The continuous injection of AFL4 suppressed tumor growth by approximately 75%. Because the discontinuation of treatment on day 7 (protocol 2) resulted in the prompt recovery of tumor growth, it is likely that VEGFR-3 is continuously required for tumor growth (Figure 4B). We also attempted to determine whether growth was suppressed if AFL4 injection was commenced from day 7 (protocol 3, Figure 4C). Although we did observe a reduction of tumor size, the effect of this protocol was not statistically significant ($P = .06$).

To examine whether AFL4 treatment suppresses the growth of other tumor types, a human prostatic cancer cell line, PC-3 was used. PC-3 cells were reported to secrete VEGF-C.[20] PC-3 cells grew more slowly than C6 cells in the subcutaneous region of the nude mice. At day14, PC-3 tumors reached an average size of 291 ± 90.1 mm^3 in control mice (Table 1), whereas we could not detect PC-3 tumor mass at day 14 in AFL4-treated mice (Table 1).

Induction of VEGFR-3 expression during tumor-induced angiogenesis

Such a dramatic suppression of tumor growth by AFL4 treatment implicates the role of VEGFR-3 in angiogenesis rather than in the formation of lymphatic vessels, though VEGFR-3 is not expressed

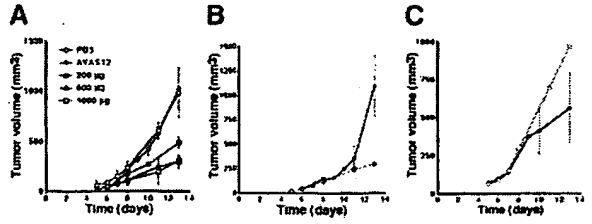

Figure 4. Anti-VEGFR-3 suppresses the growth of C6 tumor cells implanted subcutaneously in nude mice. (A) Protocol 1: days 0 to 12. Alternate-day treatment with PBS (n = 8), anti-VEGFR-2 (600 μg/dose; n = 4), or anti-VEGFR-3 (200 μg/dose, 600 μg/dose, 1000 μg/dose; n = 4 each). Tumor size at day 14 was summarized in Table 1. (B) Protocol 2: days 0 to 6. Injection of anti-VEGFR-3 treatment (600 μg/dose, closed circle; n = 4), compared with continuous injection (days 0-12) (open circle; n = 4). (C) Protocol 3: days 7 to 13. Anti-VEGFR-3 treatment (600 μg/dose, closed circle; n = 4) and PBS treatment (open circle; n = 4). The growth curves of tumors in PBS-treated mice were used as reference points for each figure.

Table 1. Suppression of tumor growth by AFL4 treatment

Antibodies	μg/dose	Tumor volume (mm^3)‡
C6 tumor*		
PBS	—	982 ± 246
Avas12†	600	1027 ± 137
AFL4	200	488 ± 58.5§
	600	296 ± 67.0§
	1000	312 ± 38.4§
PC-3 tumor		
Avas12†	600	291 ± 90.1
AFL4	600	Not detectable

Experimental procedures were as described in Figure 4.

*Results using the C6 glioblastoma cell line are the same as those presented in Figure 4.

†AVAS12 is a nonblocking mAb to mVEGFR-2.

‡Each value represents mean ± SEM of 8 mice for the PBS-treated group and 4 mice for other groups.

§$P < .01$.

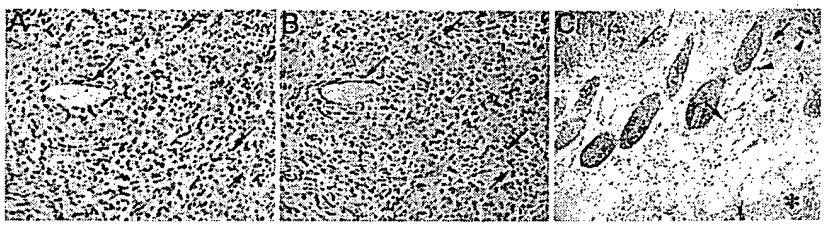

Figure 5. Induction of VEGFR-3 expression during tumor-induced angiogenesis. Immunostaining for VEGFR-3 (A) and PECAM-1 (B) in adjacent sections of C6 subcutaneous tumors (day 7) in nude mice (×200). Intratumoral VEGFR-3$^+$ (A, arrows) containing blood cells are also positive for PECAM-1 expression (B, arrows). Note that hemorrhages are not conspicuous at this stage. (C) Immunostaining for VEGFR-3 in a section of C6 tumors with surrounding tissues (×100). Note the presence of VEGFR-3$^+$ (black arrows) and VEGFR-3$^-$ (white arrows) vessels, both containing hematopoietic cells. Arrowheads indicate VEGFR-3$^+$ vessels that do not contain hematopoietic cells. Asterisk indicates the edge of a tumor.

in the blood vessels of normal tissues. Thus, we hypothesized that VEGFR-3 expression might be induced by tumor transplantation in the surrounding tissue. To test this possibility, we investigated VEGFR-3 expression in the tumor-bearing tissues. Sections of tumor and surrounding tissues were immunostained with AFL4 or anti-PECAM-1 mAb. VEGFR-3 expression was detected in intratumoral vessels (indicated by arrows) (Figure 5A). Unlike normal tissues (Figure 3E), VEGFR-3$^+$ vessels in this section contained blood cells, indicating that VEGFR-3 expression was induced in the tumor blood vessels. A similar staining pattern was seen for PECAM-1 staining of serial sections, demonstrating EC-specific expression of VEGFR-3 (Figure 5B). It should be noted, however, that not all EC in the tumor vessels expressed VEGFR-3. Intratumoral VEGFR-3$^+$ vessel density, including lymphatics, was 30 ± 1.2 per high-power field, whereas that of normal skin tissue was 7.4 ± 0.3 per high-power field. Because the intratumoral PECAM-1$^+$ vessel density was 92 ± 2.8, approximately 30% of intratumoral vessels become activated to express VEGFR-3$^+$, and because PECAM-1$^+$ vessel density in the normal skin was 38 ± 1.3, the intratumor region was indeed rich in blood vessels. VEGFR-3 staining was also induced in vessels surrounding the tumor (Figure 5C).

Histologic basis for tumor suppression in AFL4-treated mice

To gain insight into the VEGFR-3–dependent cellular processes during tumor-induced angiogenesis, we compared the vascular system surrounding tumors of AFL4-treated and control mice. In the control mouse, the size of the vascular trunk supplying branches to tumors was larger than the corresponding trunk in the tumor-free side of skin, suggesting an increase of overall blood flow in the tumor-bearing side (Figure 6A). As expected from the reduced tumor size in AFL4-treated mice, the vascular trunk governing tumor blood supply was smaller than that of the control mice (Figure 6B). In control mice, several branches of similar size stemmed from this trunk, which further divided into smaller branches (Figure 6A,C). In contrast, though the primary branches were detectable in the AFL4-treated mice, their sizes were variable, and they did not develop the fan-like architecture found in the control tumor (Figure 6B,D). Secondary and tertiary branches appeared to be very thin. Many micro-hemorrhages were found

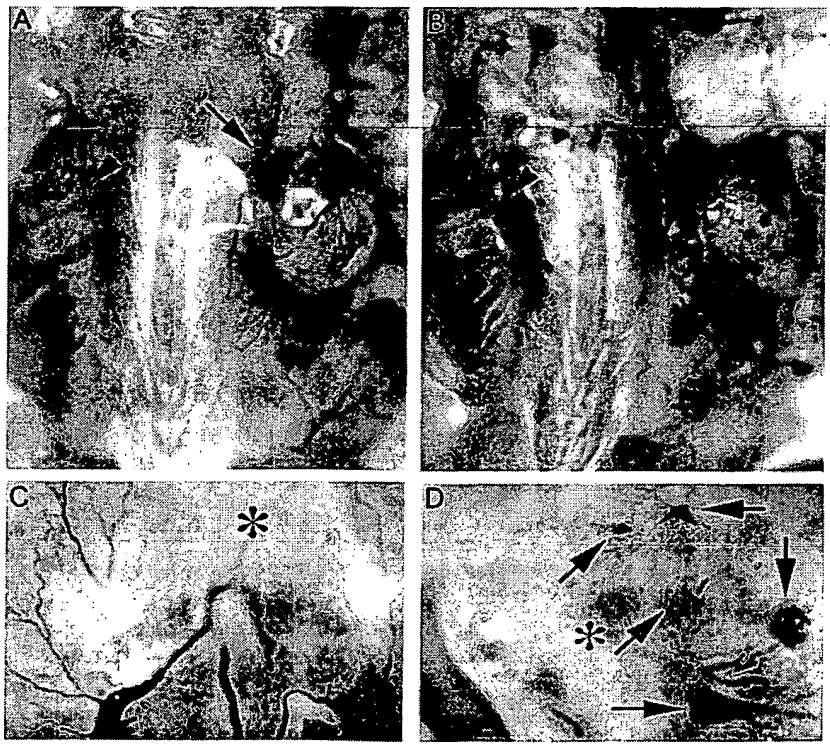

Figure 6. AFL4 treatment inhibits tumor angiogenesis. Tumor-bearing regions were photographed on day 14 after tumor transplantation. Gross appearance of representative vascularization of control (A) and AFL4-treated (B) mice. Arrows and arrowheads indicate the vascular trunks governing tumor blood supply and those of the tumor-free side, respectively. Note that the size of the trunk is larger on the tumor-bearing side than on the other side. Such a dilatation is not clearly seen in the AFL4-treated mouse. (C, D) Higher magnification than that in A and B, respectively. Secondary and tertiary branches are poorly developed in the AFL4-treated mouse. Note the many micro-hemorrhages in this AFL4-treated tumor (arrows). Asterisks indicate tumors.

along the small branches (Figure 6B,D). Although massivebleeding was frequently found in the necrotic regions of tumors in the control mice, micro-hemorrhages were rare. This macroscopic observation was confirmed by microscopic analysis. Control tumor sections stained with hematoxylin–eosin showed enlarged vessels surrounding tumors (data not shown), whereas overall vascularity around the tumors was lower in the AFL4-treated group (data not shown). Moreover, the number of PECAM-1$^+$ EC within the tumor mass was reduced to 40% in the control group (Figure 7A,B,E). Conversely, 4 times more micro-hemorrhagic regions were detected in the AFL4-treated group (Figure 7C,D,F).

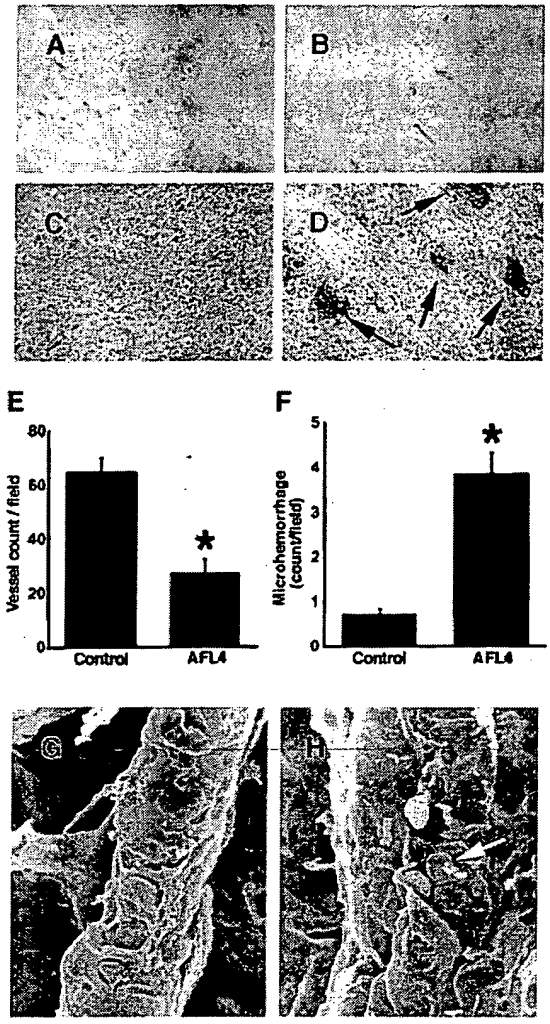

Figure 7. Histology of intratumor vasculatures. (A, B) Tumor vasculatures are visualized by anti-PECAM-1 immunostaining. Micrographs are of representative sections prepared from the control (A) and AFL4-treated mice (B) (×200). (C, D) Representative H&E-stained tumor sections from controls (C) and anti-VEGFR-3–treated mice (D) (×200). In anti-VEGFR-3–treated tumors, many micro-hemorrhages are observed (arrows). (E) Vessel counts per field were determined from at least 5 different vision fields of sections from control (n = 4; 65 ± 5.2/high-power field) and AFL4-treated mice (n = 4; 27.5 ± 5.3/high-power field) (×200). Data are plotted as mean ± SEM. *$P < .01$. (F) The number of micro-hemorrhages was scored in high-power fields (×200) of H&E-stained tumor sections (at least 5 different vision fields each of 4 tumors). Control, 0.73 ± 0.13/high-power field; AFL4, 3.9 ± 0.46/high-power field. Data are plotted as mean ± SEM. *$P < .01$. Statistical differences between groups were computed using the Student *t* test. (G) Representative SEM of a postcapillary venule in control tumors (×3800). (H) SEM of a postcapillary venule in anti-VEGFR-3–treated tumors reveals disruption of the endothelial sheet, exposing red blood cells (arrow) (×2200).

To determine the morphologic basis of the micro-hemorrhage in the AFL4-treated tissues, the vessels connecting to the tumor were analyzed systemically by SEM. We could not detect any abnormalities in the morphology of the proximal vessels of AFL4-treated mice, suggesting that these vessels developed normally (data not shown). However, at the level of postcapillary venules, disruption of the endothelial lining was observed frequently in AFL4-treated mice (Figure 7G,H). Erythrocytes could be seen through the cleft. The formation of such clefts was barely detectable in the postcapillary venule surrounding the tumors in control mice.

Discussion

In this study we established an antagonistic mAb to VEGFR-3 (AFL4) and used AFL4 to evaluate the role of this RTK in tumor angiogenesis. Although VEGFR-3 is not expressed in the vascular EC of adult mice, our result showed that VEGFR-3 expression is induced in the vascular EC upon the implantation of tumor cells. Furthermore, the growth of C6 glioma cells and PC-3 prostate carcinoma cells was suppressed by the injection of AFL4, presumably because of the inhibition of the establishment of the vascular architecture in tumor-bearing tissues. Taking the previous study on VEGFR-3$^{-/-}$ embryos[26] into account, besides embryonic angiogenesis, VEGFR-3 is involved in the neo-angiogenesis of adult tissue.

Sustained AFL4 treatment resulted in no gross anomalies in normal mice. Thus, during the 3 weeks of mAb injection, the architecture of the lymphatics and the vascular system remained unaffected in the absence of VEGFR-3 function; however, the effect over a longer period of time remains to be investigated. Recently, it has been demonstrated that the prolonged suppression of VEGF activity in the adult mouse has no effect on the maintenance of the vascular system, though it suppresses angiogenesis in the newborn mouse.[36] The fully established lymphatic and vascular systems are basically resistant to treatment with various reagents that suppress neo-angiogenesis. Because of the neo-angiogenesis–specific effect of these reagents, this approach has been expected to be chosen for cancer therapy. Our current results demonstrate clearly that VEGFR-3 is a potentially useful molecule for targeting in future cancer therapy.

Compared with the phenotype of VEGFR-2$^{-/-}$ mice, in which formation of the primitive vascular plexus is impaired,[37] it has been indicated that VEGFR-3 is involved at a later stage of vascular development, particularly in the remodeling of the primitive plexus to a higher-order architecture.[26] The absence of secondary and tertiary branches in AFL4-treated mice suggests a VEGFR-3 role in the remodeling of tumor-induced neo-angiogenesis. Angiopoietins/Tie-2 has also been implicated in the remodeling process of embryonic and tumor-induced angiogenesis. It is likely that the molecular requirements for vascular development in the embryo and for tumor-induced neo-angiogenesis are essentially the same and involve an ordered expression of multiple tyrosine kinase receptors.

Which process of angiogenesis is affected by the inhibition of VEGFR-3? Although the role of VEGFR-3 in the remodeling of vascular formation has been implicated, these reports did not specify the process beyond the word *remodeling*.[26] This may be, in part, because of an inherent difficulty in studying embryonic angiogenesis in which angiogenesis proceeds asynchronously according to region-specific timetables. In other words, various

intermediate steps of angiogenesis are mixed within an embryo. In contrast, tumor-induced angiogenesis is a synchronous process that can be induced in a relatively homogeneous microenvironment. Moreover, the progression of angiogenesis during tumor growth has been described in detail. With these considerations in mind, we attempted to obtain insight into the histologic basis of the phenotype induced by AFL4-injection. Although AFL4 treatment appeared to be identical to other anti-angiogenic reagents in that it inhibited the supply of vascular branches to the tumor, we demonstrated that micro-hemorrhage, presumably because of the disruption of the endothelial lining at the postcapillary venule level, is a characteristic feature of AFL4-treated tissues. It is difficult to rule out the possibility that this effect is caused by the cytotoxic reaction of AFL4 to VEGFR-3$^+$ EC, but we prefer to think that this disruption is derived from the blockage of VEGFR-3 function. This histologic sign has not been described in previous experiments in which other RTKs are blocked. Because micro-hemorrhages are too conspicuous to be overlooked, frequent micro-hemorrhages may be specific to VEGFR-3 inhibition.

How VEGFR-3-block caused the disruption of endothelial structure is difficult to specify. According to previous studies, sprouting of EC occurs only at the levels of capillaries, postcapillary venules, and precapillary arterioles, where no smooth muscle is present.[38] By the repeated sprouting, splitting, and anastomosis of blood vessels at this level, the overall peripheral vascular bed in the tumor-bearing tissues increases. This increase in the vascular bed contributes to the reduction of regional vascular resistance, thereby resulting in the increased blood supply. The change of blood supply induces restructuring of the more proximal vessels connecting to tumor, as observed in the current study. Because the angiopoietins/Tie2 signal was shown to regulate interactions between ECs and smooth muscle cells,[9-11] it is conceivable that this signal is required for vascular remodeling in which the distal blood vessel is restructured to the more proximal form associated with smooth muscles. In the Tie2-block experiment, however, micro-hemorrhages in tumor-bearing tissues has not been indicated. Hence, failure in interaction between ECs and smooth muscle cells may not lead to disruption of the endothelial structure.

The frequency of micro-hemorrhages found in AFL4 treated-mice suggests that AFL4 treatment inhibits maintenance of the integrity of the endothelial sheet during angiogenesis. Although further cell biology studies are required for an understanding of the underlying mechanisms, the presence of mural cells at the level of precapillary arterioles and postcapillary venules of AFL4-treated mice suggests that it may not result from inhibition of the interaction between EC and smooth muscle cells. It has been suggested that vascular permeability is increased at the site of tumor-induced angiogenesis. Thus, it is likely that the endothelial lining is agitated during neo-angiogenesis. Indeed, sprouting and pruning would imperil the integrity of the endothelial lining. Yet, micro-hemorrhage is not a frequent outcome of neo-angiogenesis, indicating that regulatory mechanisms maintain the integrity of the endothelial sheet even during dynamic restructuring of the vascular system. Therefore, we speculate that in the process of sprouting and pruning, during which integrity of the EC layer is disturbed, additional signals such as VEGFR-3 may be required for quick restoration of the EC sheet that otherwise leads to the formation of irreparable clefts. If such clefts causing micro-hemorrhage are generated during neo-angiogenesis, the rheologic resistance of the vascular system should increase, thereby resulting in the collapse of more proximal vessels as observed in the AFL4-treated mouse.

Acknowledgments

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Inhibition of VEGFR-3 Activation with the Antagonistic Antibody More Potently Suppresses Lymph Node and Distant Metastases than Inactivation of VEGFR-2

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Abstract

Lymph nodes are the first site of metastases for most types of cancer, and lymph node status is a key indicator of patient prognosis. Induction of tumor lymphangiogenesis by vascular endothelial growth factor-C (VEGF-C) has been shown to play an important role in promoting tumor metastases to lymph nodes. Here, we employed receptor-specific antagonist antibodies in an orthotopic spontaneous breast cancer metastasis model to provide direct evidence for the key role of VEGFR-3 activation in metastasis. Inhibition of VEGFR-3 activation more potently suppressed regional and distant metastases than inactivation of VEGFR-2, although VEGFR-2 blockade was more effective in inhibiting angiogenesis and tumor growth. Despite prominent proliferation, metastases were not vascularized in any of the control and treatment groups, indicating that the growth of metastases was not dependent on angiogenesis at the secondary site for the duration of the experiment. Systemic treatment with either VEGFR-2 or VEGFR-3 antagonistic antibodies suppressed tumor lymphangiogenesis, indicating that VEGFR-3 signaling affects the rate of tumor cell entry into lymphatic vessels through both lymphangiogenesis-dependent and independent mechanisms. Combination treatment with the anti-VEGFR-2 and anti-VEGFR-3 antibodies more potently decreased lymph node and lung metastases than each antibody alone. These results validate the concept of targeting the lymphatic dissemination and thereby very early steps of the metastatic process for metastasis control and suggest that a combination therapy with antiangiogenic agents may be a particularly promising approach for controlling metastases. (Cancer Res 2006; 66(5): 2650-7)

Introduction

Metastasis is the main cause of treatment failure and death for cancer patients. For most types of cancer, the first site of metastasis are lymph nodes, and the extent of lymph node involvement is a major criterion for evaluating patient prognosis and the choice of therapy (1, 2). The number of positive axillary lymph nodes, their location, and the size of lymph node metastases are evaluated to assess the stage of the disease progression. To form metastatic lesions at distant sites, cancer cells need to escape immune surveillance, survive in the blood circulation, arrest in the vasculature of the target organ, and grow at the secondary site (3). Although each step of the process represents a potential target for the antimetastatic therapy, metastatic growth is currently considered the most promising stage of the metastatic process for therapeutic targeting (4).

Angiogenesis is essential for the growth of most primary tumors and their metastases, and antiangiogenic therapy has been effective in suppressing tumorigenicity and metastases in experimental models of cancer. Vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 play a central role in tumor angiogenesis, and agents that block VEGF signaling pathways have shown promising results in clinical trials (5). Recent studies showing the ability of tumors to induce lymphangiogenesis have identified also lymphatic vessels as a potential target for the antimetastatic therapy (6). Lymphatic vessel invasion by tumor cells, increased numbers of tumor-associated lymphatics and enlarged lymphatic vessels have been frequently correlated with lymph node metastases and poor patient prognosis (2, 7). Notably, a large number of studies showed a correlation between the expression of the lymphangiogenic factor VEGF-C in human tumors and lymph node metastases (2). VEGF-C plays a key role in lymphangiogenesis by activating the VEGFR-3 receptor tyrosine kinase on lymphatic endothelial cells (1, 8–10). Mature form of VEGF-C also binds and activates VEGFR-2 (11), but whether VEGFR-2 plays a direct role in lymphangiogenesis is less clear.

Several studies in animal tumor models have provided direct evidence for the causal role of VEGF-C in tumor lymphangiogenesis and metastasis. VEGF-C has been shown to induce tumor lymphangiogenesis and facilitate tumor spread to the regional lymph nodes in mouse models of breast (12–14), pancreatic (15), gastric (16), and colorectal cancer (17). Our previous studies in a mouse xenograft model of breast cancer showed increase of not only lymph node but also distant metastases upon VEGF-C overexpression (12). Here, we evaluated the VEGF-C/VEGFR-3 signaling pathway as a target for the antimetastatic therapy and have compared the effectiveness of antiangiogenesis versus anti-lymphangiogenesis approach for control of tumor metastases. Our results show that inhibition of VEGFR-3 signaling with the function-blocking antibody mF4-31C1 was more effective in suppressing metastases than inactivation of VEGFR-2, and that the combination therapy was more potent in suppressing metastases than single-antibody treatments. These results show a key role for VEGFR-3 activation in metastasis and validate the concept of targeting the lymphatic endothelium and lymphatic spread for metastasis control.

Note: N. Roberts and B. Kloos contributed equally to this work.

Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Spontaneous metastasis assay. MDA-MB-435/GFP cells (18) transfected with the human VEGF-C cDNA (cl.13) or pcDNA/control vector (cl.2) have been established and cultured as previously described (12) and will be referred to as MDA/VEGF-C and MDA/pcDNA, respectively. Cells were injected bilaterally into the second mammary fat pads of athymic, female, 8-week-old NCR *nu/nu* mice (1×10^6/100 μL, serum-free culture medium). Tumor growth was monitored weekly. Upon sacrifice, mice were perfused through the heart with PBS, and lungs, liver, left and right axillary, and brachial lymph nodes were harvested and processed for evaluation of metastases by flow cytometry as described below. For analysis of green fluorescent protein (GFP)–labeled metastases in tissue sections, tissues were processed as described previously (12), embedded in OCT and frozen. Tumors were embedded in OCT and frozen for immunostaining or snap-frozen in liquid nitrogen for protein extractions.

Tail vein metastasis assay. MDA/pcDNA or MDA/VEGF-C cells were injected i.v. into the lateral tail vein of 8-week-old NCR female, athymic *nu/nu* mice (1×10^6/100 μL HBSS). Mice were sacrificed after 10 weeks, and lymph nodes, lungs, and liver were collected and processed for evaluation of metastases.

Blocking antibodies. Neutralizing rat monoclonal antibodies specific for mouse VEGFR-3 (mF4-31C1; ref. 19) and mouse VEGFR-2 (DC101; refs. 20, 21) were generated by ImClone Systems Inc. (New York, NY). The mF4-31C1 antibody has been shown to block VEGF-C–induced phosphorylation of mouse VEGFR-3 and VEGF-C–induced cell proliferation *in vitro* and to inhibit physiologic regeneration of lymphatic vessels *in vivo* (19).

Treatment modalities. Animals were randomly assigned to one of the four treatment groups (mF4-31C1, DC101, mF4-31C1+DC101, and control), 10 mice per group. In the prevention regimen, treatment was initiated at the time of orthotopic tumor cell injection. In the intervention regimen, treatment commenced when tumors and metastases were established, 4 weeks after the orthotopic tumor cell inoculation. Blocking antibodies were administered i.p. at 800 μg/mouse every second day, over the 6-week period. For the combination treatment, both antibodies were administered concurrently at 800 μg/mouse.

Quantification of metastases by flow cytometry. Tissue dissociation, sample preparation, and fluorescence-activated cell sorting (FACS) analysis were done essentially as described (22), with some modifications. Upon sacrifice, organs were collected and dissociated individually by mechanical disruption and incubation with 400 units/mL collagenase D (Roche, Indianapolis, IN) for 30 minutes at 37°C. Collagenase activity was stopped with 10 mmol/L EDTA, and samples were passed through a 70-μm cell strainer (BD Biosciences, Bedford, MA). Cell suspensions were centrifuged and incubated in ice-cold 0.17 mol/l NH_4Cl for 10 minutes to lyse RBC. Finally, cells were washed with HBSS, stained with 3 μg/mL propidium iodide, and resuspended in FACS buffer (1%fetal bovine serum/PBS).

Samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Entire samples of each lymph node were analyzed. Total number of cells in lymph nodes typically ranged from 5×10^4 to 2×10^6. At least 2×10^6 cells were analyzed in each lung or liver sample. Background fluorescence was determined by using tissue from mice that were not implanted with tumor cells. Nonviable cells were excluded based on propidium iodide staining. Data acquisition and analysis were done with Cell Quest software (BD Biosciences). Tumor burden in organs was calculated as the percentage of fluorescent tumor cells detected in the total population of viable cells in the sample. As few as 1 fluorescent tumor cell in 1×10^5 host cells (0.001% total cells) could be accurately detected. Taking into consideration the minimal number of cells in lymph nodes and the sensitivity of detection, <1 fluorescent cell in 1×10^4 host cells (<0.01%) was considered negative.

Immunofluorescent staining. Cryosections (~7 μm) of tumors, lymph nodes, and lungs were stained as previously described (23), using antibodies against mouse CD31 and CD34 (1:50; BD Biosciences PharMingen, San Diego, CA), LYVE-1 (1:800; United Biomedical, Inc., Hauppauge, NY), VEGFR-3 (1:40; R&D Systems, Minneapolis, MN), VEGFR-2 (1:100; R&D Systems), Ki67 (1:1,000; Novocastra, Newcastle, United Kingdom), and corresponding secondary antibodies labeled with Alexa Fluor 488 or 594 (Molecular Probes, Eugene, OR). Cell nuclei were counterstained with 10 μg/mL Hoechst bisbenzimide (Sigma-Aldrich, St. Louis, MO). Specimens were examined with a Nikon E-600 microscope (Nikon, Melville, NY), and images were captured with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI).

Quantification of tumor vasculature. Tumor sections were double-stained with antibodies to LYVE-1 and CD31, to visualize lymphatic (LYVE-1$^+$) and blood vessels (LYVE-1$^-$/CD31$^+$). Computer-assisted morphometric analysis of digital images was done using the IPLab software (Scanalytics, Fairfax, VA). Five tumors were examined in each experimental group, and five areas of each tumor were evaluated at ×20 magnification. Lymphatic and blood vasculatures were quantified in the tumor areas with the highest density of respective vessels ("hotspots"). Total vessel area was calculated per tumor, and data were expressed as the average vessel area of five tumors, or as the percentage of the tumor area occupied with the vasculature. The average vessel size was also determined for each tumor.

Statistical analyses. The data were analyzed using a mixed model ANOVA, to take into account correlation structure in the data (four lymph nodes in each mouse). Because the outcome variables of percent GFP$^+$ cells and the area of the vasculature both exhibited right-skewed distributions, these variables were log-transformed before analysis. The least-squares means for each treatment group were back-transformed to the original scale to provide the geometric means. The *P*s for the differences between the means of treatment group versus control were calculated using Dunnett's adjustment for multiple comparisons of treatment means versus a single control. The Tukey-Kramer–adjusted *P*s were used for the comparisons between the different treatments. All statistical analyses were done using SAS statistics software (SAS Institute, Inc., Cary, NC).

Results

Tumor metastasis model. We have previously shown that VEGF-C overexpressed in MDA-MB-435/GFP human breast cancer cells promotes tumor metastasis to lymph nodes and to lungs 12 weeks after the orthotopic injection of tumor cells (Table 1; Fig. 1; ref. 12). To determine the onset of metastasis in this tumor model, we have analyzed the kinetics of tumor dissemination by a highly sensitive flow cytometry–based assay for detection of GFP-labeled cells in tissues. Flow cytometric analysis showed that the metastasis of MDA/VEGF-C cells to regional lymph nodes is an early event, with tumor cells detectable in the lymph nodes of most animals after 1 week (8 of 10), and in all animals within the 3 weeks (10 of 10) after the orthotopic injection. In contrast, MDA/pcDNA cells had disseminated to lymph nodes in only 50% of the mice (5 of 10) by 12 weeks, indicating that VEGF-C significantly accelerates the metastatic process. Earlier onset of metastasis was independent of tumor size, as both tumor cell lines showed comparable growth rates *in vivo*, reaching an average tumor volume of ~35 mm^3 within 3 weeks after tumor cell injection.

Lung metastases were also formed early, with tumor cells detectable in the lungs of 80% of the mice 1 week after the orthotopic injection. By 3 weeks, lung metastases were established in 100% of the mice. To determine at which step in the metastatic process is VEGF-C/VEGFR-3 signaling important for promoting dissemination to distant sites, we compared the ability of MDA/pcDNA and MDA/VEGF-C cells to form colonies in the target organs after i.v. injection. Both cell lines metastasized to the lungs and liver at the same frequency 10 weeks after the inoculation (Table 2). Interestingly, tumor cells also colonized lymph nodes after the i.v. injection; however, no difference was observed between the control and VEGF-C–overexpressing cells (Table 2). These results show that, whereas VEGF-C clearly promotes dissemination from the primary tumor, once tumor cells are

Table 1. Effects of treatments with mF4-31C1 and DC101 antibodies on the incidence of lymph node metastases

	Prevention				Intervention			
	pcDNA, PBS	VEGF-C, PBS	VEGF-C, 31C1	VEGF-C, DC101	VEGF-C, PBS	VEGF-C, 31C1	VEGF-C, DC101	VEGF-C, 31C1+DC101
No. mice with positive LN	5/10	10/10	3/10	8/10	8/10	7/10	8/9	5/10
No. positive LN	5/39	24/40	6/39	21/40	32/38	24/38	30/36	14/40
% Positive LN	13	60	15	53	84	63	83	35

NOTE: Tumor cells were injected orthotopically, and blocking antibodies were given systemically at 800 μg/mL, every 2 days. Antibodies were administered over the 6-week period commencing at the time of tumor cell inoculation (prevention regimen), or 4 weeks after the tumor cell injection (intervention regimen).
Abbreviation: LN, lymph node.

present in the bloodstream, VEGF-C does not provide an advantage for the formation of metastases in distant organs.

Systemic treatment with antagonist antibody to VEGFR-3 potently inhibits lymph node metastases. To evaluate the role of VEGFR-3 in tumor dissemination, we examined the effects of systemic inhibition of VEGFR-3 activation on metastasis of MDA/VEGF-C cells in two different treatment regimens. In the prevention regimen, blocking antibody mF4-31C1 that is specific for mouse VEGFR-3 was administered concurrently with the tumor cell injection. In the intervention regimen, the antibody treatment commenced 6 weeks after the tumor cell injection, when metastases were established. Systemic treatment with the mF4-31C1 antibody significantly reduced the incidence of lymph node metastases in the prevention regimen (Table 1). In fact, the effect of VEGF-C on promoting tumor metastases was completely abolished with the anti-VEGFR-3 antibody treatment, as the incidence of lymph node metastases in mF4-31C1–treated mice bearing MDA/VEGF-C tumors was comparable with that of control mice bearing tumors devoid of VEGF-C expression (Table 1). Quantitative assessment of metastases by flow cytometry showed that the number of tumor cells in the lymph nodes of mice treated with the anti-VEGFR-3 antibody was reduced by 74% as compared with the control-treated mice (% GFP^+ tumor cells in lymph nodes: 0.0092 ± 0.0344 versus 0.035 ± 0.0836, respectively; $P < 0.05$; Fig. 1*A*). The low average number of tumor cells reflects mainly the lesser number of lymph nodes containing tumor cells in anti-VEGFR-3–treated mice. The number of tumor cells in the lymph nodes that contained the metastatic cells, however, was comparable between the control and anti-VEGFR-3–treated mice (0.058 ± 0.1 versus 0.06 ± 0.068, respectively; $P > 0.05$).

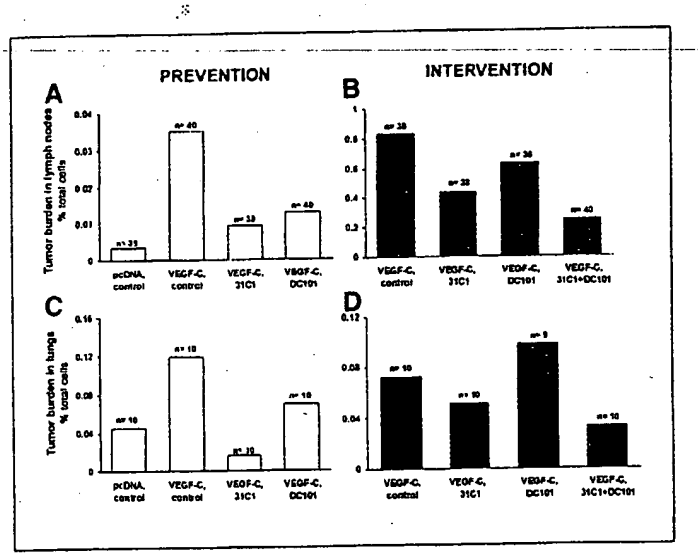

Figure 1. Quantification of tumor burden in lymph nodes and lungs following treatment with mF4-31C1 and DC101 antibodies. Tumor cells were inoculated orthotopically, and metastatic tumor burden was quantified by flow cytometry. *Columns*, average number of fluorescent tumor cells, expressed as the percentage of total cells (negative samples included).

In the intervention treatment regimen, mF4-31C1 antibody lowered tumor burden in the lymph nodes by 47% (Fig. 1*B*). The number of mice with lymph node metastases was comparable between the mF4-31C1–treated and control group, but the number of lymph nodes with metastases in each mouse was lower in the anti-VEGFR-3–treated group (Table 1).

Systemic treatment with the anti-VEGFR-3 blocking antibody inhibits lung metastases. Blocking antibody to VEGFR-3 drastically reduced tumor burden in the lungs in the prevention and less prominently in the intervention treatment regimen (Fig. 1*C* and *D*). In the prevention regimen, blocking VEGFR-3 decreased tumor burden in the lungs by 87% compared with the control (% GFP^+ tumor cells: 0.016 ± 0.01 versus 0.119 ± 0.12, respectively; $P < 0.05$). The effect of VEGF-C on promoting lung metastases was abolished with the anti-VEGFR-3 antibody treatment. In the intervention regimen, the inhibition was much less pronounced (%GFP^+ tumor cells: mF4-31C1 treated 0.05 ± 0.07 versus control 0.07 ± 0.1; $P > 0.05$).

Blocking VEGFR-2 is less effective in halting metastases than blocking VEGFR-3. Inhibition of VEGFR-2 signaling had only minor effect on the incidence of metastases to lymph nodes in the prevention regimen and no effect in the intervention treatment regimen (Table 1). However, anti-VEGFR-2 blocking antibody DC101 decreased overall tumor burden in the lymph nodes by 64% in the prevention regimen (control, 0.035 ± 0.0836 versus DC101, 0.0128 ± 0.019; $P > 0.05$), although the effect was not statistically significant. In the intervention regimen, blocking VEGFR-2 also did not show a significant effect (control, 0.833 ± 1.51 versus DC101, 0.633 ± 0.88; $P > 0.05$; Fig. 1*A* and *B*). Because the number of lymph nodes with metastases was comparable between the control and anti-VEGFR-2–treated mice, reduced

Table 2. VEGF-C has no effect on organ metastases in experimental metastasis model

	Experimental metastases (i.v.)		
	Lymph node	Lung	Liver
MDA/pcDNA	0.9 ± 0.5	6.7 ± 2.3	6.7 ± 3.1
MDA/VEGF-C	0.8 ± 0.6	5.2 ± 1.6	6.4 ± 2.3

NOTE: MDA/pcDNA or MDA/VEGF-C cells were injected i.v., and metastases were quantified after 10 weeks. Tumor burden is expressed as the percentage of total cells (mean ± SD) in lymph nodes (n = 28), lungs (n = 7), or liver (n = 7).

tumor burden in lymph nodes of DC101-treated mice reflects primarily the lower number of tumor cells in lymph nodes with metastases. Indeed, the average number of metastatic cells in lymph nodes was decreased by 59% with the DC101 treatment (control, 0.058 ± 0.1 versus DC101, 0.024 ± 0.02; $P < 0.05$).

Blocking VEGFR-2 also proved less effective in reducing lung metastases. In the prevention regimen, DC101 antibody decreased lung tumor burden, by 42% (%GFP$^+$ tumor cells: control, 0.119 ± 0.12 versus DC101, 0.069 ± 0.082; $P > 0.05$) compared with 87% decrease of lung tumor burden with the mF4-31C1 antibody (Fig. 1*C*). In the intervention regimen, the extent of lung metastases was comparable between control and DC101-treated groups (%GFP$^+$ tumor cells: 0.07 ± 0.102 versus 0.1 ± 0.084, respectively; $P > 0.05$; Fig. 1*D*).

Blocking of either VEGFR-3 or VEGFR-2 inhibits tumor lymphangiogenesis. Our previous studies showed that overexpression of VEGF-C in MDA-MB-435/GFP cells resulted in increased intratumoral lymphangiogenesis and enlargement of peritumoral lymphatic vessels (12). Thus, we investigated whether the different effects of VEGFR-3 and VEGFR-2 blocking on tumor metastasis can be attributed to distinct roles of these two receptors in tumor lymphangiogenesis. Lymphatic vessels were visualized by immunostaining with the antibody for LYVE-1, a specific marker of lymphatic endothelium (24, 25). Blocking VEGFR-3 activation resulted in the complete normalization of peritumoral lymphatic vasculature (Fig. 2). In contrast to the hyperplastic lymphatic vessels observed around the tumors of control animals, lymphatic vessels surrounding tumors of mice treated with mF4-31C1 were small, compressed, and indistinguishable from the lymphatic vessels in normal skin. Moreover, anti-VEGFR-3–treated mice displayed complete inhibition of intratumoral lymphangiogenesis compared with control-treated mice (Fig. 3); intratumoral lymphatic vessel densities were decreased by 95% in the prevention and in the intervention treatment regimen ($P < 0.001$). Notably, blocking VEGFR-2 also resulted in the normalization of peritumoral lymphatic vasculature as well as in the significant reduction of intratumoral lymphangiogenesis (Fig. 2). DC101 antibody decreased intratumoral lymphatic vessel densities by 81% ($P < 0.001$) in both treatment regimens (Fig. 3).

As determined by immunostaining, VEGFR-2 was strongly expressed by peritumoral lymphatic vessels (Fig. 4). However, in contrast to VEGFR-3 that was present on all peritumoral and intratumoral lymphatics, VEGFR-2 was weakly expressed or absent from the intratumoral lymphatic vessels (Fig. 4).

Relative potency of blocking VEGFR-2 or VEGFR-3 on tumor angiogenesis and growth. Anti-VEGFR-3 antibody reduced blood vessel densities and decreased the size of tumor blood vessels. Quantitative analysis showed a 69% reduction of the tumor area occupied with the blood vessels (% tumor area: control, 4.7 ± 1.37% versus mF4-31C1, 1.45 ± 0.48%; $P < 0.0001$). Likewise, DC101 antibody decreased the total blood vessel area in tumors by 71% (control, 4.7 ± 1.37% versus DC101, 1.36 ± 0.39%; $P < 0.0001$). However, whereas in the DC101-treated group large tumor areas were completely avascular and the remaining vessels seemed large and mature, in the mF4-31C1–treated group, small blood vessels were scattered throughout the tumor (Fig. 5*A-D*). The average blood vessel size in the mF4-31C1–treated group was significantly smaller than in the DC101-treated group (DC101, 8,631 ± 2,170 μm^2 versus mF4-31C1, 2,846 ± 930 μm^2; $P < 0.001$). Results were comparable in both treatment regimens. As determined by

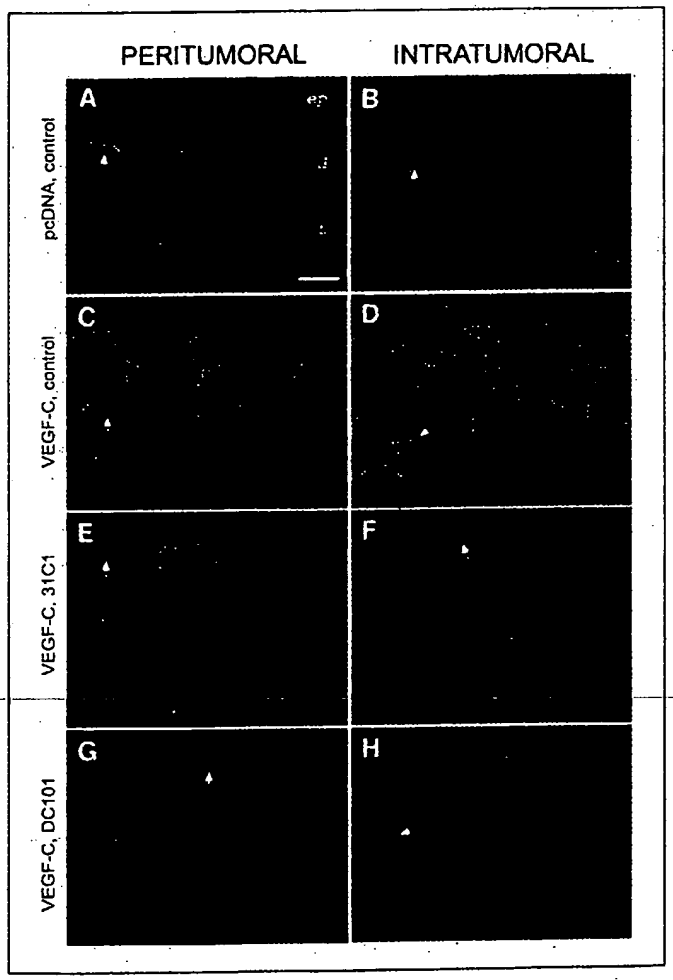

Figure 2. Blocking VEGFR-3 or VEGFR-2 inhibits tumor lymphangiogenesis. Immunofluorescent staining for LYVE-1 (*green, arrows*) revealed small, mostly collapsed lymphatic vessels in the skin overlaying control-transfected tumors (*A*) and a few intratumoral lymphatic vessels (*B*). The skin adjacent to the MDA/VEGF-C tumors was characterized by highly enlarged lymphatics (*C*), and the tumors were extensively infiltrated throughout with the lymphatic vessels (*D*). In contrast, lymphatic vessels at the periphery of MDA/VEGF-C tumors treated with the anti-VEGFR-3 antibody (mF4-31C1) had returned to their normal size (*E*), and very few intratumoral lymphatics were detected (*F*). Likewise, tumors treated with the anti-VEGFR-2 antibody (DC101) were surrounded with small lymphatics (*G*), and the intratumoral lymphatic vessel density was dramatically decreased (*H*). Note that the appearance of lymphatic vessels in the mF4-31C1 (*E* and *F*) and DC101-treated tumors (*G* and *H*) is comparable with the control (*A* and *B*). Cell nuclei are counterstained with Hoechst (*blue*). *ep*, epithelium; *d*, dermis; *t*, tumor. Bar, 100 μm.

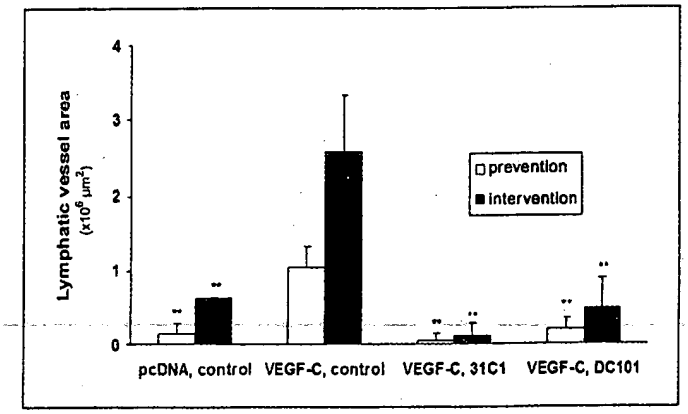

Figure 3. Quantification of tumor lymphangiogenesis. Computer-assisted image analysis showed that mF4-31C1–treated mice displayed nearly complete inhibition of tumor lymphangiogenesis compared with control-treated mice; intratumoral lymphatic vessel area was also markedly decreased with the DC101 treatment yet to a somewhat lesser extent than with the mF4-31C1 treatment. The results were comparable between the prevention (*open columns*) and the intervention (*filled columns*) treatment regimen. Because mice were sacrificed later in the intervention then in the prevention experiment (10 versus 6 weeks, respectively), the overall lymphatic vessel densities are always higher in the intervention group. *Columns*, mean lymphatic vessel area ($n = 5$ for each treatment group); *bars*, SD. **, $P < 0.01$.

immunostaining, VEGFR-3 and VEGFR-2 were expressed by the subpopulation of tumor blood vessels (Fig. 4).

Tumor growth rate was moderately attenuated with the anti-VEGFR-3 treatment (Fig. 5*E*). In the prevention regimen, blocking VEGFR-3 resulted in the 45% decrease of tumor volume 6 weeks after the tumor cell injection. Blocking VEGFR-2 reduced tumor volume by 84%. In the intervention regimen, anti-VEGFR-3 antibody diminished tumor size by 35%, but the difference was not statistically significant. Because differences in tumor size may result from changed expression of VEGFs, we examined the effects of blocking VEGFR-3 or VEGFR-2 on expression of VEGFs in tumors. As determined by Western analysis, expression levels of VEGF-A, VEGF-C, or VEGF-D were not altered with the antibody treatments (data not shown).

Phenotypic characteristics of metastases. To gain insight into the mechanism by which inactivation of VEGFR-2 and VEGFR-3 signaling suppresses metastases, we evaluated lymph node and lung metastases by histology and examined metastatic nodules for proliferation and angiogenesis. At the time points examined (i.e., 6 weeks for the prevention and 10 weeks for the intervention experiments), we have not observed any macrometastases on the surface of the organs examined. Micrometastases, which were detected in the lymph node sections by GFP fluorescence, were localized predominantly in the subcapsular zone. Typical appearance of metastases in the lymph nodes is shown in Supplementary Fig. S1. The size of lymph node metastases varied, from only few cells per section to different size nodules ranging from 100 to 400 μm in diameter (Supplementary Fig. S1*A-H*). For comparison, the size of lymph nodes enlarged by the presence of a tumor ranged from 1 to 3 mm in diameter. Most commonly, the metastatic nodules were composed entirely of viable cells, as indicated by the uniform expression of GFP (Supplementary Fig. S1*A* and *B, E-H*). In larger metastases, necrosis was apparent in the center of the nodule, and tumor cell proliferation was restricted to the outer cell layers (Supplementary Fig. S1*C* and *D*; Fig. 6*A* and *B*). There were no obvious differences in the appearance of lymph node metastases among the different antibody treatments. Typical histology of a large lymph node metastasis is shown in Fig. 6*A*. Unexpectedly, we found that all lymph node metastases, including the largest nodules in the nontreated group, were avascular, as shown by the CD31 and CD34 immunostaining (Fig. 6*C* and *D*). Tumor cell proliferation in the lymph node was apparently high, as shown by Ki67 labeling (Fig. 6*B*). Similarly, even the largest lung metastases were not vascularized yet clearly showed proliferative activity (Fig. 6*E-G*). Upon different antibody treatments, both the size and the number of the metastatic lesions were decreased (Supplementary Fig. S2*A-D*). Although the appearance and the size of the metastatic lesions were rather heterogeneous within the same group of specimens (Supplementary Fig. S2*E* and *F*), the phenotype of metastases was not notably different between the control and the treated groups.

Combination treatment is more effective in suppressing metastases than single antibody treatments. The effects of the combined treatment with the anti-VEGFR-2 and anti-VEGFR-3

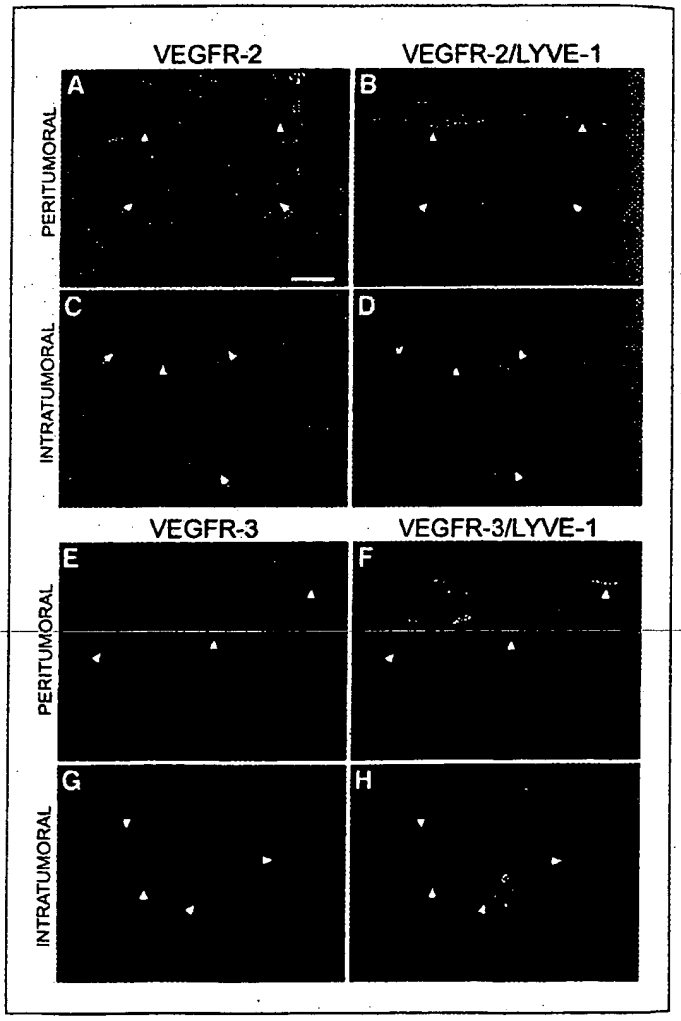

Figure 4. Expression of VEGFR-2 and VEGFR-3 on tumor lymphatic and blood vasculature. *A-D*, double immunofluorescent staining for LYVE-1 (*green*) and VEGFR-2 (*red*) showed that VEGFR-2 is strongly expressed on all peritumoral lymphatic vessels (*arrowheads; A* and *B*) but is partially down-regulated or absent from the intratumoral lymphatics (*C* and *D*). *E-H*, in contrast, VEGFR-3 is strongly expressed by all peritumoral (*E* and *F*) and intratumoral (*G* and *H*) lymphatic vessels (*arrowheads*). A fraction of tumor-associated blood vessels (*arrows*) expressed VEGFR-2 (*A-D*) or VEGFR-3 (*E* and *H*). MDA/VEGF-C control tumors. Cell nuclei are counterstained with Hoechst (*blue*). *ep*, epithelium; *d*, dermis; *t*, tumor. Bar, 100 μm.

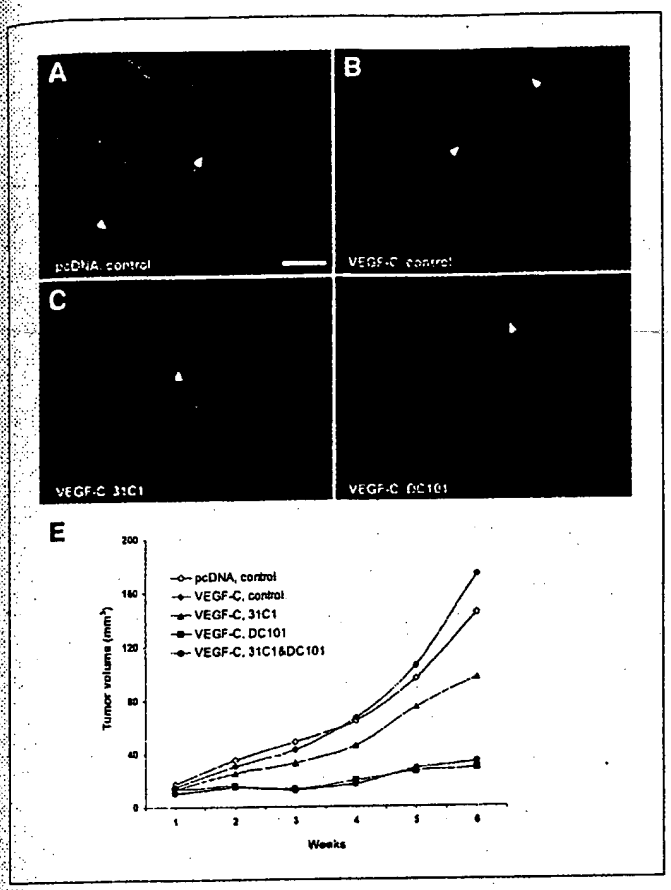

Figure 5. Effects of treatments with mF4-31C1 and DC101 antibodies on tumor angiogenesis and growth. The density of intratumoral blood vessels ($CD31^+$/$LYVE\text{-}1^-$) was comparable between the MDA/pcDNA (*A*) and MDA/VEGF-C tumors (*B*). Blocking VEGFR-3 (*C*) or VEGFR-2 (*D*) decreased tumor angiogenesis. DC101-treated tumors were characterized by large tumor areas devoid of the vasculature, and the remaining blood vessels seemed large and mature (*D*). In contrast, blood vessels in mF4-31C1–treated tumors were small and scattered throughout the tumor (*C*). Bar, 100 μm. *E*, tumor growth rates were not significantly different between the MDA/pcDNA and MDA/VEGF-C cells. Tumor growth was delayed significantly with the mF4-31C1 treatment ($P < 0.001$), albeit less dramatically than the with the DC101 antibody treatment ($P < 0.001$). Combination treatment was comparable with the DC101 alone. *Points*, average size of 20 tumors (10 mice, 2 tumors each; prevention regimen).

antibodies on metastases were evaluated in the intervention treatment regimen. Combining the DC101 and mF4-31C1 antibodies very prominently reduced the incidence of lymph node metastases (Table 1). The number of mice with metastases and the number of lymph nodes with metastases in each mouse were both significantly lower in the combined treatment group compared with the control. Moreover, combined treatment was more effective than single treatment with the anti-VEGFR-2 or anti-VEGFR-3 antibodies. Combination treatment also decreased the overall tumor burden in the lymph nodes by 70% (control, 0.833 ± 1.51 versus DC101+mF4-31C1, 0.25 ± 0.47; $P = 0.05$; Fig. 1*B*). Lung tumor burden was decreased by 54% (%GFP^+ tumor cells: control, 0.07 ± 0.1 versus DC101+mF4-31C1, 0.033 ± 0.03; $P > 0.05$), the combination being more effective than mF4-31C1 single treatment (Fig. 1*D*). In addition, only 6 of 10 mice developed lung metastases in the double-treated group compared with 9 in 10 mice in the control.

Combination of DC101 and mF4-31C1 antibodies decreased tumor angiogenesis by 79% (compared with control; $P < 0.0001$), which was slightly more potent than with the each antibody alone (69% and 71% inhibition with mF4-31C1 and DC101 antibody, respectively; $P < 0.01$). Lymphangiogenesis was not inhibited more than with the single-antibody treatments. Finally, the combined treatment was not more effective in inhibiting tumor growth than blocking VEGFR-2 alone (Fig. 5*E*).

Discussion

In this study, we evaluated the specific roles of VEGFR-3 versus VEGFR-2 signaling in metastasis and show that the selective inhibition of VEGFR-3 signaling is more effective in suppressing regional and distant tumor metastases than inhibition of VEGFR-2. Our previous studies have shown that MDA-MB-435 breast cancer cells producing high levels of VEGF-C induce tumor lymphangiogenesis, and that the VEGF-C–mediated activation of tumor-associated lymphatic vessels facilitates metastases in a mouse xenograft model (12). By using this tumor model, we show here that the systemic treatment with the function-blocking antibody mF4-31C1, which selectively inhibits murine VEGFR-3 signaling (19), potently reduced tumor cell dissemination to the lymph nodes, indicating that the activation of VEGFR-3 signaling is critical for lymphogenous spread. Notably, the blockade of VEGFR-3 signaling also reduced lymph node metastases when the antibody was administered after the metastases were established, indicating for the first time a potential use of lymphatic vessel targeting for halting metastases after the tumor dissemination has occurred and not only as a prevention strategy.

The soluble form of VEGFR-3 has been reported to prevent formation of lymph node metastases in several experimental tumor

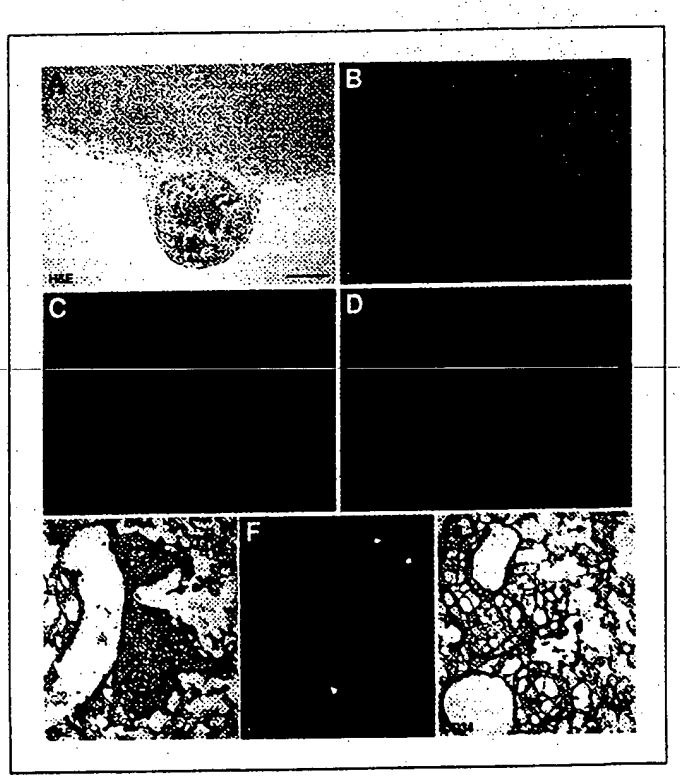

Figure 6. Growth and vascularization of metastatic lesions in the serial sections of lymph nodes (*A-D*) and lungs (*E-G*). *A*, H&E staining of a representative large metastatic lesion in the subcapsular region of the lymph node. *B*, Ki67 immunostaining showed extensive proliferation at the periphery of the metastatic lesion and the central necrotic region. Immunostaining with CD31 (*C*) and CD34 (*D*) showing vasculature surrounding but not infiltrating the metastatic lesion. *E*, H&E staining of a typical, large metastatic lesion in the lung. *F*, Ki67 immunostaining (*arrows*) showing tumor cell proliferation. *G*, CD34 immunostaining (*arrows*) showing that lesion is not vascularized. Bar, 100 μm.

models. Adenoviral administration of soluble VEGFR-3-Ig fusion protein reduced incidence of lymph node metastases from lung, melanoma, and prostate tumors implanted s.c. in immunodeficient mice (26, 27). In another study, VEGFR-3-Ig overexpressed in breast cancer cells suppressed formation of lymph node metastases in immunocompetent rats (14). Most recently, small interfering RNA (siRNA)–mediated VEGF-C gene silencing has been shown to inhibit tumor lymphangiogenesis and metastasis (28). Because soluble VEGFR-3 and the VEGF-C siRNA approach interfere with the activation of both VEGFR-2 and VEGFR-3, these studies could not assess the specific role of each receptor in metastasis. It has been reported that the monoclonal antibody AFL4 specifically blocks murine VEGFR-3 signaling and inhibits corneal lymphangiogenesis (29). In experimental tumor models, AFL4 has been found to inhibit angiogenesis and tumor growth (30) and to suppress lymph node metastases (31). In contrast, Pytowski et al. reported that AFL4 binds to VEGFR-3 but does not block VEGFR-3 signaling and have suggested that the observed effects of AFL4 may be mediated by nonantagonist mechanisms, such as the steric hindrance of VEGFR-3 dimerization or antibody-induced reduction in surface receptor expression (19). In our own studies, we have not observed any effects of the AFL4 antibody on tumor lymphangiogenesis or metastasis.[3] In view of the above, the results presented in this report provide the first direct evidence for the pivotal role of VEGFR-3 signaling in lymphogenous metastasis.

Importance of VEGFR-3 signaling in metastases to lymph nodes is further underscored by our findings that blocking VEGFR-2 was less effective in halting metastases then blocking VEGFR-3, although VEGFR-2 blockade more potently inhibited angiogenesis and primary tumor growth. Because blocking of VEGFR-2 mainly affected angiogenesis and tumor growth, we hypothesized that inactivation of VEGFR-2 suppresses metastases by restraining metastatic growth, whereas VEGFR-3 inactivation mainly inhibits tumor spread. To test this hypothesis, we analyzed lymph node and lung metastases for angiogenesis and proliferation. We found, to our surprise, that despite prominent proliferation, metastases were not vascularized in any of the control and treatment groups, indicating that the growth of metastases was not dependent on angiogenesis at the secondary site within the duration of the experiment. This could explain the limited efficacy of the antiangiogenic therapy with the DC101 antibody for metastasis control in this experimental model. It is conceivable that the metastatic cells were not highly sensitive to the antiangiogenesis treatment because the metastatic lesions were too small to depend on angiogenesis for growth. It has also been suggested in the literature that the vascular dependence of malignant cells may be heterogeneous, particularly, that in the late stages of tumor progression vascular demand may be lowered (32–34). Furthermore, in well-vascularized organs, such as lungs, the dependence on angiogenesis may be diminished because of the extensive preexisting vascular network which can be co-opted by the metastatic cells (35, 36). Hence, the observed suppression of metastases with the DC101 antibody could be due to the significant decrease in the size of a primary tumor and decreased lymphangiogenesis, which may limit the rate of tumor cell escape from the tumor.

Effects of VEGFR-3 inhibition on metastases could be explained, at least in part, by inhibition of lymphangiogenesis. We and others have shown that increased lymphangiogenesis and recruitment of lymphatic vessels into the tumor increase the propensity of tumors to metastasize (12–15, 17, 37). Accordingly, decreasing the number of lymphatic vessels in the vicinity of tumor cells may restrain tumor spread by restricting tumor cell access to the lymphatic vasculature. Because VEGFR-2 blockade also inhibited tumor lymphangiogenesis but without decreasing the incidence of lymph node metastases, the reduction of lymphatic vessel densities alone can not explain the suppression of metastases by VEGFR-3 inactivation, raising a question about the mechanism by which VEGF-C and VEGFR-3 activation promote tumor dissemination. We have proposed previously that activation of lymphatic endothelium by VEGF-C may facilitate tumor cell entry into the lymphatics by promoting molecular interactions between tumor cells and lymphatic endothelium (12, 38, 39). Activation of lymphatic endothelial cells by VEGF-C and other VEGFR-3 ligands may facilitate the escape of cancer cells from the primary tumor by promoting release of chemokines that may attract tumor cells into the lymphatics or by altering adhesive properties of lymphatic endothelium to support tumor invasion.

In support of this concept, high levels of VEGF-C expression have been associated with lymphatic vessel invasion and lymph node metastases in the multitude of human tumors, whereas tumor metastases have not always been associated with increased lymphangiogenesis (2). In the present study, we have observed fewer lymphatic vessels infiltrated with tumor cells in tumors treated with the anti-VEGFR-3 antibodies compared with anti-VEGFR-2–treated tumors. Furthermore, our *in vitro* studies showed that lymphatic endothelial cells potently promote tumor cell chemotaxis.[4] Taken together, these results suggest that in addition to lymphangiogenesis, activation of lymphatic vessels via the VEGFR-3 pathway may facilitate tumor metastases by promoting entry of tumor cells into the peripheral lymphatics.

To gain insight into the mechanism by which VEGF-C/VEGFR-3 signaling promotes distant metastases, the ability of control and VEGF-C–overexpressing tumor cells to form colonies in target organs was assessed in the experimental metastasis assay. In this assay, which models later stages of the metastatic process, there was no difference in the colonization of lungs or liver between control and VEGF-C–expressing cells. These findings show that VEGF-C does not provide an advantage for tumor cell survival in the bloodstream, arrest, extravasation, or growth at the secondary site and further support the concept that VEGF-C increases formation of distant metastases via the lymphatic pathway. Nevertheless, a possibility remains that VEGF-C may also facilitate tumor cell invasion of the blood vasculature at the primary tumor site. Interestingly, when injected directly into the blood circulation, tumor cells also entered lymph nodes. In this case, tumor burden in the lymph nodes was not increased by VEGF-C, reinforcing the conclusion that VEGF-C does not increase growth of metastases in lymph nodes but facilitates tumor cell arrival from the primary tumor.

In summary, we show that selective inhibition of the VEGF-C/VEGFR-3 signaling pathway is an effective strategy for control of regional and distant metastases. It has been suggested that targeting the late steps of the metastatic process, such as growth of metastases at the secondary site, may be the most effective

[3] N. Roberts and M. Skobe, unpublished data.

[4] S.K. Das and M. Skobe, unpublished data.

antimetastatic strategy (4, 40). The present study validates the concept of targeting the lymphatic endothelium, and thereby very early stages of the metastatic process, for metastasis control. Our results, showing the combination treatment with mF4-31C1 and DC101 antibodies to be more potent in suppressing metastases than either treatment alone are encouraging and suggest a potential promise for strategies which combine targeting both tumor dissemination and growth for the treatment of metastatic disease.

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Hyperplasia of Lymphatic Vessels in VEGF-C Transgenic Mice

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No growth factors specific for the lymphatic vascular system have yet been described. Vascular endothelial growth factor (VEGF) regulates vascular permeability and angiogenesis, but does not promote lymphangiogenesis. Overexpression of VEGF-C, a ligand of the VEGF receptors VEGFR-3 and VEGFR-2, in the skin of transgenic mice resulted in lymphatic, but not vascular, endothelial proliferation and vessel enlargement. Thus, VEGF-C induces selective hyperplasia of the lymphatic vasculature, which is involved in the draining of interstitial fluid and in immune function, inflammation, and tumor metastasis. VEGF-C may play a role in disorders involving the lymphatic system and may be of potential use in therapeutic lymphangiogenesis.

The four known members of the VEGF family, VEGF (*1*), VEGF-B (*2*), VEGF-C (*3*, *4*), and platelet-derived growth factor (*5*) have different roles as regulatory factors of endothelia (*6*). In order to clarify the function of VEGF-C in vivo, its cDNA was cloned between the human keratin 14 (K14) promoter and polyadenylation signal for expression in transgenic mice (*7*). The K14 promoter has been shown to target gene expression to the basal cells of stratified squamous epithelia (*8*). Of the 27 mice analyzed at 3 weeks of age, three were transgene-positive, having approximately 40 to 50, 20, and 4 to 6 copies, respectively, of the transgene in their genome. The latter two mice transmitted the gene to two of 40 and six of 11 pups, respectively.

The transgenic mice were small and had slightly swollen eyelids and poorly developed fur. Histological examination showed that the epidermis was hyperplastic and the number of hair follicles was reduced; these effects were considered secondary to other phenotypic changes (*9*). The dermis was atrophic, and its connective tissue was replaced by large, dilated vessels devoid of red cells but lined with a thin endothelial cell layer (Fig. 1, A and B). Such abnormal vessels were confined to the dermis and resembled the dysfunctional, dilated spaces characteristic of hyperplastic lymphatic vessels (*10*). In addition, the ultrastructural features were reminiscent of lymphatic vessels, which differ from blood vessels in that they have overlapping endothelial junctions, anchoring filaments in the vessel wall, and a discontinuous or even partially absent basement membrane (Fig. 1C) (*11*). Antibodies to collagen types IV and XVIII (*12*), and laminin gave little or no staining of the vessels, whereas the basement membrane staining of other vessels was prominent (Fig. 1D) (*13*). The endothelium was also characterized by positive staining with monoclonal antibodies to desmoplakins I and II, expressed in lymphatic, but not in vascular endothelial cells (*14*). Collectively, these findings suggest that the abnormal vessels are of lymphatic origin.

Abundant VEGF-C mRNA was detected in the epidermis and hair follicles of the transgenic mice (Fig. 2, A and B), whereas mRNAs encoding its receptor VEGFR-3 (*15*) and VEGFR-2 (*16*), as well as the Tie-1 endothelial receptor tyrosine kinase (*17*), were expressed in endothelial cells lining the abnormal vessels (Fig. 2, C and E) (*13*). In the skin of littermate control animals, VEGFR-3 was detected only in the superficial subpapillary layer of lymphatic vessels, whereas VEGFR-2 was observed in all endothelia (Fig. 2, D and F), in agreement with earlier findings (*18*, *19*).

The lymphatic endothelium has a great capacity to distend in order to adapt to functional requirements. To ascertain whether vessel dilation was due to endothelial distension or proliferation, we carried out in vitro proliferation assays. The VEGF-C receptor interaction in the transgenic mice apparently transduces a mitogenic signal, because in contrast to littermate controls, the lymphatic endothelium of the skin from young K14–VEGF-C mice showed increased DNA synthesis, as demonstrated by bromodeoxyuridine (BrdU) incorporation followed by staining with antibodies to BrdU (Fig. 3, A and B).

Angiogenesis is a multistep process that includes endothelial sprouting, migration, and proliferation (*20*). To estimate the contribution of such processes to the transgenic phenotype, we analyzed the morphology and function of the lymphatic vessels, using fluorescent microlymphography (*21*). A typical honeycomb-like network with similar mesh sizes was detected in both control and transgenic mice (Fig. 3, C and D), but the diameter of the vessels in transgenic

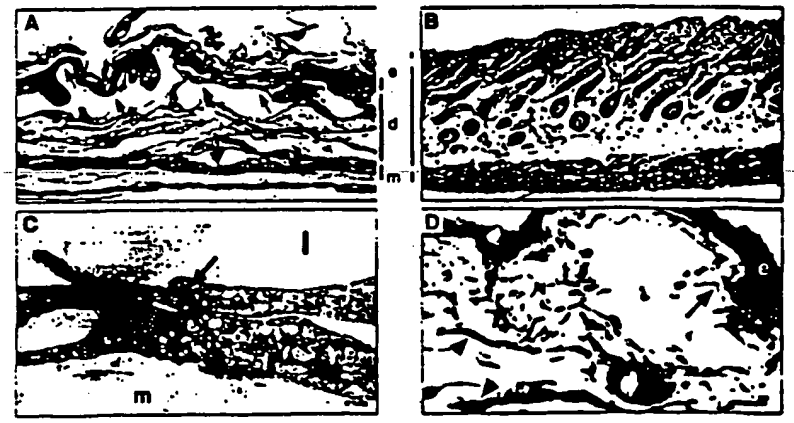

Fig. 1. Analysis of the skin of transgenic mice. (A) Hematoxylin-eosin stained section of the skin of a 2-month-old transgenic mouse. Hyperkeratotic epidermis (e) showed underlying vessel spaces lined with endothelium (arrows) but devoid of red cells (compare with the dermal vein shown with an arrowhead). The dermis (d) is atrophic compared with the control littermate skin (B) (45% versus 65% of the dermal thickness, respectively), and the muscle layer (m) is also reduced. (C) Electron microscopy shows the endothelial junctions of an abnormal vessel (arrow) (*25*); l, lumen; m, mesenchyme. In (D), the basal lamina is stained for type XVIII collagen in veins (arrowheads) but not in the lymphatic endothelium (arrow) (*26*). Scale bars for (A) and (B), 250 μm; (C), 100 nm; and (D), 25 μm.

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mice was approximately twice that of controls (Table 1). Some dysfunction of the abnormal vessels was indicated by the fact that it took longer for the dextran to completely fill the abnormal vessels. Injection of fluorescein isothiocyanate (FITC)–dextran into the tail vein, followed by fluorescence microscopy of the ear showed that the blood vascular morphology was unaltered and leukocyte rolling and adherence appeared normal (Fig. 3, E and F) (*13*). Thus, the endothelial proliferation induced by VEGF-C leads to hyperplasia of the superficial lymphatic network, but does not induce the sprouting of new vessels.

These effects of VEGF-C overexpression are unexpectedly specific, particularly as VEGF-C is also capable of binding to and activating VEGFR-2, which is the major mitogenic receptor of blood vessel endothelial cells (*16*). In culture, high concentrations of VEGF-C stimulate the growth and migration of bovine capillary endothelial cells that express VEGFR-2, but not significant amounts of VEGFR-3 (*3*). In addition, VEGF-C induces vascular permeability in the Miles assay, presumably by its effect on VEGFR-2 (*22*). In vivo, the specific effects of VEGF-C on lymphatic endothelial cells may reflect a requirement for the formation of VEGFR-3 × VEGFR-2 heterodimers for endothelial cell proliferation at physiological concentrations of the growth factor. Such possible heterodimers may help to explain how three homologous VEGFs exert partially redundant, yet strikingly specific biological effects.

In summary, VEGF-C appears to induce specific lymphatic endothelial proliferation and hyperplasia of the lymphatic vascul

Table 1. Structural parameters of lymphatic and blood vessel networks for transgenic and control mice. Mesh size describes vessel density. Diameters and mesh sizes are in micrometers (mean ± SD).

Parameter	Transgenic	Control	*P* value*
	Lymphatic vessels†		
Diameter	142.3 ± 26.2	68.2 ± 21.7	0.014
Horizontal mesh size	1003 ± 87.1	960.8 ± 93.1	0.220
Vertical mesh size	507.3 ± 58.9	488.8 ± 59.9	0.540
	Blood vessels‡		
Median diameter	8.3 ± 0.6	7.6 ± 1.1	0.121
Vessel density (cm/cm²)	199.2 ± 6.6	216.4 ± 20.0	0.301

*Mann-Whitney test. †Number of animals for transgenic and control groups were 4 and 5, respectively. ‡Number of animals for transgenic and control groups were 3 and 6, respectively.

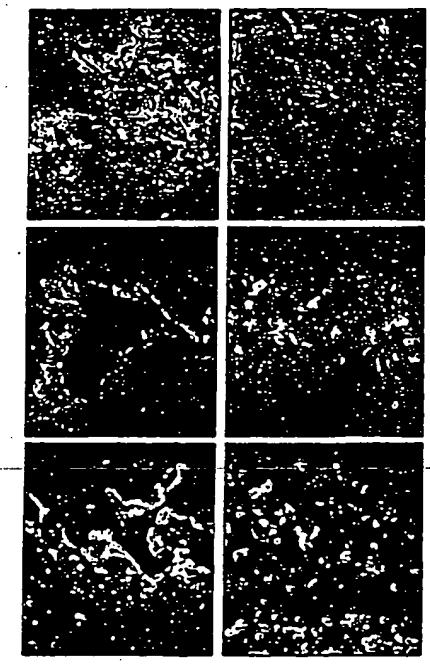

Fig. 2. In situ hybridization analysis of the skin of K14-VEGF-C transgenic mice. (**A**) and (**B**) show hybridization of transgenic skin with VEGF-C antisense and sense probes. (**C**) and (**D**) and (**E**) and (**F**) show in situ hybridization for VEGFR-3 and VEGFR-2, respectively, in the skin of transgenic and littermate control animals (*27*). Arrows in (A) indicate basal keratinocytes; in (C) through (E), they point out the lymphatic endothelium; and in (F), arrows show endothelial cells. Scale bar, 20 μm.

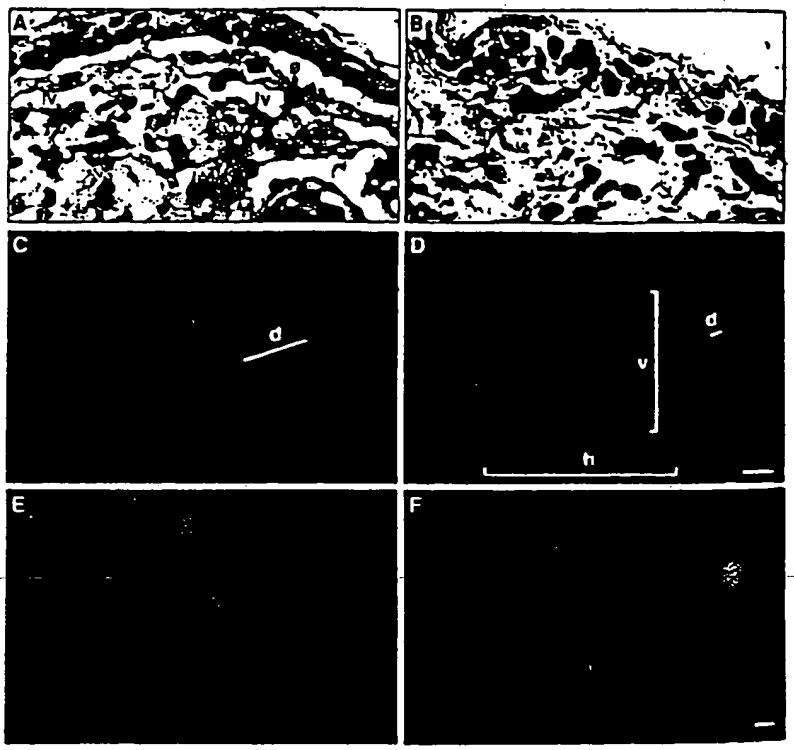

Fig. 3. Immunohistochemical analysis of endothelial proliferation and intravital fluorescence microlymphography and microvessel angiography. (**A**) and (**B**) show staining of cells in the S phase of the cell cycle, through BrdU incorporation into DNA and its immunohistochemical detection (*28*). In 2-week-old transgenic mice, the nuclear staining was observed in many endothelial cells of the lymphatic vessels (V) as well as in keratinocytes [red arrows in (A)]. In nontransgenic littermates, mainly nuclei of keratinocytes of epidermis and some dermal cells are stained [red arrows in (B)]; unstained nuclei were observed in both cases (green arrows). Ø marks artefactual detachment of the epidermis during sample preparation. (**C**) and (**D**) illustrate the lymphatic vessels of transgenic and control skin, respectively, through fluorescence microscopy after intradermal injection of FITC-dextran (*21*, *29*). The measured parameters are diameter (d) [in (C) and (D)] and horizontal (h) and vertical (v) mesh sizes [in (D) only]. Blood vessels of the ear after injection of FITC-dextran into the tail vein of transgenic and control mice are shown in (E) and (F), respectively (*24*). Scale bars for (A) and (B), 5 μm; (C) and (D), 250 μm; (E) and (F), 1 mm.

in vivo. So far, there is no evidence that tumors can stimulate the growth of new lymphatic vessels (23), but further studies should establish the role of VEGF-C in lymphangiomas and in tumor metastasis via the lymphatic vasculature as well as in various other disorders involving the lymphatic system and their treatment.

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7. The human VEGF-C cDNA (GenBank accession number X94216) was blunt-end ligated to the Bam HI restriction site of the K14 expression cassette (*8*), and an Eco RI–Hind III fragment containing the K14 promoter, VEGF-C cDNA, and K14 polyadenylation signal was isolated and injected into fertilized oocytes of the FVB/NIH strain of mice. The injected zygotes were transplanted into oviducts of pseudopregnant C57BL/6 × DBA/2J hybrid mice. We analyzed the resulting founder mice for the presence of the transgene by polymerase chain reaction of tail DNA, with the primers: 5′-CATGTACGAACCGCCAG-3′ and 5′-AATGACCAGAGAGAGGCGAG-3′. The tail DNAs were also subjected to endonuclease digestion, Southern blotting, and hybridization analysis using the transgene fragment as the probe.
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25. For electron microscopy, tissue pieces from two transgenic skin biopsies were fixed in formaldehyde, postfixed in 2% osmium tetroxide, and embedded in LX 112. Sagittal ultrathin sections were studied with a Jeol1200Ex electron microscope.
26. For immunohistochemistry, we stained cryostat sections of 5 to 10 μm from abdominal and back skin with anti-mouse desmoplakin I and II monoclonal antibodies (Progen), affinity-purified rabbit anti-mouse collagen XVIII IgG (a gift from T. Pihlajaniemi), and rabbit anti-mouse laminin IgG (provided by E. Lehtonen), by using the Vectastain ABC Elite kit (Vector Laboratories). Normal mouse or rabbit sera were used as negative controls for the stainings.
27. In situ hybridization of sections was performed as described [A. Kaipainen *et al.*, *J. Exp. Med.* **178**, 2077 (1993)]. The human VEGF-C antisense RNA probe was generated from linearized pCR™II plasmid (Invitrogen) containing an Eco RI fragment corresponding to nucleotides 628 through 1037 of human VEGF-C cDNA. The VEGFR-3 probe was described earlier (*18*). The VEGFR-2 probe was an Eco RI fragment covering base pairs 1958 through 2683 (GenBank accession number X59397, a gift from J. Rossant).
28. For measurement of DNA synthesis, small (3 mm by 3 mm) skin biopsies from four transgenic and four control mice were incubated in Dulbecco's modification of Eagle's medium with 10 μg/ml BrdU for 6 hours at 37°C, fixed in 70% ethanol for 12 hours, and embedded in paraffin. After a 30-min treatment with 0.1% pepsin in 0.1 M HCl at room temperature to denature DNA, staining was carried out as above with mouse monoclonal antibodies to BrdU (Amersham).
29. Fluorescence microlymphography was performed as follows. The staining of the lymphatic network *in vivo* was carried out as described (*20*). Briefly, 8-week-old mice were anesthetized and placed on a heating pad to maintain a 37°C temperature. A 30-gauge needle, connected to a catheter filled with a solution of FITC-dextran (2 MD, 5 mg/ml in phosphate-buffered solution), was injected intradermally into the tip of the tail. The solution was infused with a constant hydrostatic pressure equivalent to a 50-cm column of water (flow rate averaging roughly 0.01 μl/min) until the extent of network filling remained constant (approximately 2 hours). Flow rate and fluorescence intensity were monitored continuously throughout the experiment. The intravital fluorescence microscopy of blood vessels was as described (*24*).
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Geometric Control of Cell Life and Death

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Human and bovine capillary endothelial cells were switched from growth to apoptosis by using micropatterned substrates that contained extracellular matrix-coated adhesive islands of decreasing size to progressively restrict cell extension. Cell spreading also was varied while maintaining the total cell-matrix contact area constant by changing the spacing between multiple focal adhesion-sized islands. Cell shape was found to govern whether individual cells grow or die, regardless of the type of matrix protein or antibody to integrin used to mediate adhesion. Local geometric control of cell growth and viability may therefore represent a fundamental mechanism for developmental regulation within the tissue microenvironment.

The local differentials in cell growth and viability that drive morphogenesis in complex tissues, such as branching capillary networks (*1*, *2*), are controlled through modulation of cell binding to extracellular matrix (ECM) (*3*–*6*). Local disruption of ECM by pharmacologic or genetic means results in selective programmed cell death (apoptosis) within adjacent cells (*2*, *6*, *7*). Soluble integrin $\alpha_V\beta_3$ antagonists also induce apoptosis in cultured endothelial cells and promote capillary involution in vivo (*8*). Furthermore, death can be prevented by allowing suspended cells to attach to immobilized antibodies to integrins or by inhibiting tyrosine phosphatases (*7*, *9*). For these reasons, adhesion-dependent control of apoptosis has been assumed to be mediated by changes in integrin signaling. Analysis of capillary regression in vivo, however, has revealed that dying capillary cells remain in contact with ECM fragments, thus suggesting that the cell foreshortening caused by ECM dissolution may be the signal that initiates the death program (*2*). This possibility is supported by the finding that endothelial cells spread and grow on large (>100-μm diameter) microcarrier beads (*4*), whereas they rapidly die when bound to small (4.5 μm) ECM-coated beads (*10*) that cluster integrins and activate signaling but do not support cell extension (*11*).

Understanding how this apoptotic switch is controlled in capillary cells has enormous clinical implications, because angiogenesis is a prerequisite for tumor growth (*12*). Thus, we set out to determine whether cell shape or integrin binding per se governs life and death in these cells. We first measured apoptosis rates in suspended

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VEGF and VEGF-C: Specific Induction of Angiogenesis and Lymphangiogenesis in the Differentiated Avian Chorioallantoic Membrane

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The lymphangiogenic potency of endothelial growth factors has not been studied to date. This is partially due to the lack of *in vivo* lymphangiogenesis assays. We have studied the lymphatics of differentiated avian chorioallantoic membrane (CAM) using microinjection of Mercox resin, semi- and ultrathin sectioning, immunohistochemical detection of fibronectin and α-smooth muscle actin, and *in situ* hybridization with VEGFR-2 and VEGFR-3 probes. CAM is drained by lymphatic vessels which are arranged in a regular pattern. Arterioles and arteries are accompanied by a pair of interconnected lymphatics and form a plexus around bigger arteries. Veins are also associated with lymphatics, particularly larger veins, which are surrounded by a lymphatic plexus. The lymphatics are characterized by an extremely thin endothelial lining, pores, and the absence of a basal lamina. Patches of the extracellular matrix can be stained with an antibody against fibronectin. Lymphatic endothelial cells of differentiated CAM show ultrastructural features of this cell type. CAM lymphatics do not possess mediae. In contrast, the lymphatic trunks of the umbilical stalk are invested by a single but discontinuous layer of smooth muscle cells. CAM lymphatics express VEGFR-2 and VEGFR-3. Both the regular pattern and the typical structure of these lymphatics suggest that CAM is a suitable site to study the *in vivo* effects of potential lymphangiogenic factors. We have studied the effects of VEGF homo- and heterodimers, VEGF/PlGF heterodimers, and PlGF and VEGF-C homodimers on Day 13 CAM. All the growth factors containing at least one VEGF chain are angiogenic but do not induce lymphangiogenesis. PlGF-1 and PlGF-2 are neither angiogenic nor lymphangiogenic. VEGF-C is the first lymphangiogenic factor and seems to be highly chemoattractive for lymphatic endothelial cells. It induces proliferation of lymphatic endothelial cells and development of new lymphatic sinuses which are directed immediately beneath the chorionic epithelium. Our studies show that VEGF and VEGF-C are specific angiogenic and lymphangiogenic growth factors, respectively.

INTRODUCTION

The development of blood vessels (angiogenesis) has been studied extensively during the past years, whereas the development of lymphatic vessels (lymphangiogenesis) has gained relatively little attention, despite the clinical relevance of lymphangiogenesis. It has been suggested to be a key process linking lymphological syndromes such as lymphedema, lymphangiectasia, lymphangioma, and lymphangiosarcoma (Witte and Witte, 1986).

Since the introduction of the chorioallantoic membrane (CAM) assay (Folkman, 1974), this method has often been used to study effects of potential angiogenic factors *in vivo*. In a modified version of the CAM assay employing differentiated CAM (Wilting *et al.*, 1991, 1992), the vascular endothelial growth factor (VEGF) (Senger *et al.*, 1983; Connolly *et al.*, 1989; Leung *et al.*, 1989) has been shown to be a highly specific angiogenic factor. However, it has rarely been noted that CAM is drained by a dense network of lymphatic vessels, although the existence of these vessels has already been described by Budge (1887). CAM therefore appears to be a suitable site to study the effects of both potential angiogenic and lymphangiogenic factors.

Secreted proteins highly homologous to VEGF have recently been found. Among these, placenta growth factor (PlGF) shares 40% identity with VEGF (Maglione *et al.*,

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1991). The function of PlGF is unknown. VEGF-B, which is highly expressed in skeletal muscle and heart, possesses 43% identity with VEGF (Olofsson *et al.*, 1996). Its function has yet to be identified. Furthermore, a growth factor with about 30% identity with VEGF has been named VEGF-C (Joukov *et al.*, 1996). VEGF-C appears to be a potential lymphangiogenic factor, as both VEGF-C receptors, VEGFR-2 and VEGFR-3 (Joukov *et al.*, 1996), are expressed in lymphatic endothelial cells (Kaipainen *et al.*, 1995; Wilting *et al.*, 1996, 1997a).

As a prerequisite for studying the effect of potential lymphangiogenic factors, the morphology and pattern of the lymphatics of normal CAM must be described. We have therefore studied the lymphatics of differentiated CAM of chick and quail embryos using semi- and ultrathin sectioning, immunohistochemical staining with anti-α-smooth muscle actin and fibronectin antibodies, *in situ* hybridization with VEGFR-2 and VEGFR-3 probes, and Mercox injection. We aimed to determine whether VEGF and PlGF homo- and heterodimers and VEGF-C homodimers affect both blood vascular and lymphatic endothelial cells.

MATERIAL AND METHODS

Embryos

Fertilized eggs of the White Leghorn chick (*Gallus gallus*) and the Japanese quail (*Coturnix coturnix*) were incubated at 37.8°C and 80% humidity. Differentiated CAM of 16-day-old chick and 13-day-old quail embryos was studied with the following methods.

Histology

Specimens were fixed in 3% glutaraldehyde and 2% formaldehyde in 0.12 *M* sodium cacodylate buffer, postfixed with 1% osmium solution, immersed with uranyl acetate, and embedded in Epon resin (Serva, Heidelberg, Germany). Semithin (0.75 μm) and ultrathin (70 nm) sections were cut with an Ultracut S (Leika, Bensheim, Germany). Ultrathin sections were studied with an EM 10 (Zeiss, Stuttgart, Germany).

Immunohistochemistry

Specimens were fixed in Serra's solution (Serra, 1946), dehydrated, and embedded in Durcupan resin (Fluka, Buchs, Switzerland). Polymerization was performed at 40°C. Semithin sections (1.5 μm) were collected on silan-coated slides. The resin was dissolved with 20% sodium ethoxide (Merck, Munich, Germany) and the sections were rinsed with ethanol and bleached with methanol/H_2O_2. Anti-α-smooth muscle actin antibody (Sigma, Deisenhofen, Germany), diluted 1:5000, was used as first antibody. Peroxidase-conjugated goat anti-mouse IgG (Sigma; 1:200) was used as second antibody and DAB as chromogen. The B3/D6 fibronectin antibody (Gardner and Fambrough, 1983) was purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA) and diluted 1:500. Second antibody and DAB staining were described above.

Injection Method

The lymphatics of 16-day-old chick embryos were perfused with glutaraldehyde/formaldehyde fixative. Two milliliters of Mercox-blue (Norwald, Hamburg, Germany) was mixed with 25 μl of accelerator and injected into the lymphatics of CAM using fine glass needles and a micromanipulator.

In Situ Hybridization

In situ hybridization was performed on quail tissue with *Quek1*/VEGFR-2 and *Quek2*/VEGFR-3 probes (Eichmann *et al.*, 1993, 1996). The probes were cloned into pcDNA/Amp (Invitrogen, San Diego). The linearized antisense probes were 3.0 (*Quek1*) and 1.5 kb (*Quek2*) in length. Specimens were fixed in Serra's solution, dehydrated, and embedded in paraffin. Eight-micrometer sections were mounted on silan-coated slides, dewaxed, and hybridized at 65°C overnight. After washing, the sections were incubated with an alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (1:4000) at 4°C overnight. After washing, nitroblue tetrazolium and X-phosphate (Boehringer, Mannheim, Germany) were used as chromogens to reveal a blue signal. The sense probes were used as controls and did not show a signal (Wilting *et al.*, 1997a).

CAM Assay

On Day 4 of development, a window was made into the egg shell of chick eggs. The embryos were checked for normal development and the eggs sealed with cellotape. They were further incubated until Day 13. Thermanox coverslips (Nunc, Naperville, IL) were cut into disks of about 5 mm in diameter. Sterile and salt-free growth factors were dissolved in distilled water and about 3.3 μg/5 μl was pipetted on the disks. After air-drying, the inverted disks were applied on CAM. After 3 days, the specimens were fixed in 3% glutaraldehyde and 2% formaldehyde and rinsed in 0.12 *M* sodium cacodylate buffer. They were photographed with a stereomicroscope (Wild M8) and embedded for semi- and ultrathin sectioning as described above. Controls were performed with carrier disks alone.

Growth Factors

The following human recombinant growth factors were applied on CAM of 13-day-old chick embryos: $VEGF_{121}$ (46 experiments), $VEGF_{165}$ (29), $VEGF_{121/165}$ heterodimer (27), PlGF-1 (11), PlGF-2 (14), $VEGF_{121}$/PlGF-1 heterodimer (33), and $VEGF_{165}$/PlGF-2 heterodimer (6). Production of the growth factors in *Escherichia coli* and subsequent purification and dimerization have been described recently (Birkenhäger *et al.*, 1996). VEGF-C (25) was produced in insect cells. The endogenous signal sequence for secretion was exchanged by that of melittin. The N- and C-termini that are proteolytically processed *in vivo* to obtain biological activity were omitted (Kukk *et al.*, 1996; Joukov *et al.*, 1996, 1997). All growth factors were desalted with a PD-10 column (Pharmacia, Uppsala, Sweden) and lyophilized.

Proliferation Studies

Proliferation of blood vascular and lymphatic endothelial cells was monitored with the BrdU/anti-BrdU method (Gratzner, 1982) after application of $VEGF_{121}$ and VEGF-C on CAM of 13-day-old chick embryos. One and two days later, the embryos were incu-

bated with 100 μl of a 40 m*M* BrdU solution (Sigma) for 45 min. The specimens were then fixed with ethanol containing 3% acetic acid. Paraffin sections were stained as previously described by Wilting *et al.* (1994).

RESULTS

Pattern of CAM Lymphatics

Mercox injections into the lymphatics were performed in the region of the allantoic stalk. From here it was possible to fill both the intraembryonic lymphatics and those of CAM. A pair of lymphatics was found along the arteries and arterioles of CAM (Fig. 1a). Along the arteries, the pair of lymphatics was regularly interconnected by lymphatic capillaries, and blind-ending extensions were also visible (Fig. 1b). Furthermore, lymphatics were regularly detected along veins. A dense network of lymphatics was observed surrounding the largest veins (Fig. 1c). Where the arteries and veins were running in parallel, lymphatics were usually located in the angle between them. The CAM lymphatics were continued to the embryo by collecting ducts along the chorioallantoic artery and vein in the allantoic stalk (Fig. 1d). In the embryo, the lymphatic ducts were connected with the paired thoracoabdominal trunks (thoracic ducts) reaching right and left venous angles.

Structure of CAM Lymphatics

In semithin sections, the lymphatics could be distinguished from blood vessels because of their extremely thin endothelial lining (Fig. 2a). Pores were regularly present between lymphatic endothelial cells. Formation of a media was not observed. This was confirmed in ultrathin sections (Fig. 2b). Here it was noted that lymphatic endothelial cells possessed lamellipodia in both luminal and abluminal directions (Figs. 2b and 2c). Focal contacts were observed connecting the cells. In the cytoplasm smooth and rough endoplasmic reticulum, free ribosomes, Golgi apparatus, and numerous mitochondria were observed (Fig. 2d). Furthermore, coated pits and vesicles with different inclusions were present. Bundles of filaments were found in some areas (Fig. 2d). A basal lamina was not present, but patches of extracellular matrix were visible. These patches could be stained with an antibody against fibronectin. Small amounts of fibronectin were present throughout the stroma of CAM. Higher amounts were detected in the media of veins (Fig. 2e).

The presence of smooth muscle cells of CAM lymphatics was also studied in semithin sections using an antibody against α-smooth muscle actin. Smooth muscle cells were found in all parts of the vascular tree except for the capillaries. The lymphatics of CAM did not possess a media (Fig. 3a). In contrast, media formation was observed in the region of the allantoic stalk. Here the collecting lymphatic ducts possessed a single but discontinuous layer of smooth muscle cells (Figs. 3b–3d).

Receptors of CAM Lymphatics

The expression of VEGFR-2 and VEGFR-3 in differentiated CAM of 13-day-old quail embryos was studied with digoxigenin-labeled RNA probes. In a previous study (Wilting *et al.*, 1996) it was shown that VEGFR-2 was expressed in a subpopulation of arterial endothelial cells and in accompanying vessels, which appeared to be lymphatics. This result could be confirmed (Fig. 4a). The vascular endothelial cells of the intraepithelial capillaries were negative. Furthermore, we observed expression of VEGFR-2 in a subpopulation of venous endothelial cells. Endothelial cells of the accompanying lymphatic plexus were also positive (Fig. 4b). Therefore, in differentiated CAM, a subpopulation of arterial and venous endothelial cells and those of the lymphatics expressed VEGFR-2.

In accordance with previous studies (Wilting *et al.*, 1996) we did not observe expression of VEGFR-3 in endothelial cells of arteries and capillaries (Fig. 4c). Our additional studies on CAM veins showed that these were also negative (Fig. 4d). However, a signal was detected in endothelial cells of vessels which were located adjacent to arteries and veins, indicating that these were lymphatics (Figs. 4c and 4d). Therefore, the combination of both VEGFR-2 and VEGFR-3 was only observed in lymphatic endothelium of differentiated CAM.

Effects of Endothelial Growth Factors

The angiogenic potential of different VEGF splice forms on differentiated CAM has been documented (Wilting *et al.*, 1992, 1993, 1996). The effects of PlGF homodimers, VEGF/PlGF heterodimers, and VEGF-C homodimers have not been studied in detail, and, except for $VEGF_{121}$, the potential lymphangiogenic potency of growth factors has not been studied (Wilting *et al.*, 1996).

VEGF and PlGF homo- and heterodimers were produced in *E. coli* (Birkenhäger *et al.*, 1996) and about 3.3 μg of each factor was applied on CAM of 13-day-old chick embryos. The macroscopically visible effects are shown in Fig. 5; the corresponding semithin sections are shown in Fig. 6. In the controls, no alterations of the vascular pattern were visible (Fig. 5a). The capillary plexus was located in the chorionic epithelium, and all larger conducting and draining vessels with the accompanying lymphatics were found in the stroma of CAM (Fig. 6a). $VEGF_{121}$ induced angiogenesis. Formation of new capillaries was seen in the precapillary area of the vasculature (Fig. 5b). A great number of capillaries penetrated the stroma of CAM, which normally does not contain capillaries (Fig. 6b). The lymphatics appeared not to be affected. We did not observe sprouting or dilatation of the lymphatics in the application area (Fig. 6b). The same results were obtained with $VEGF_{165}$ and the heterodimer $VEGF_{121/165}$. Both forms of the growth factor induced new capillaries, whereas the lymphatics were not affected (Figs. 5c, 5d, 6c and 6d). A different result was obtained with PlGF-1 and PlGF-2. Angiogenesis and lymphangiogenesis were not detected either macroscopically (Figs. 5e and 5f)

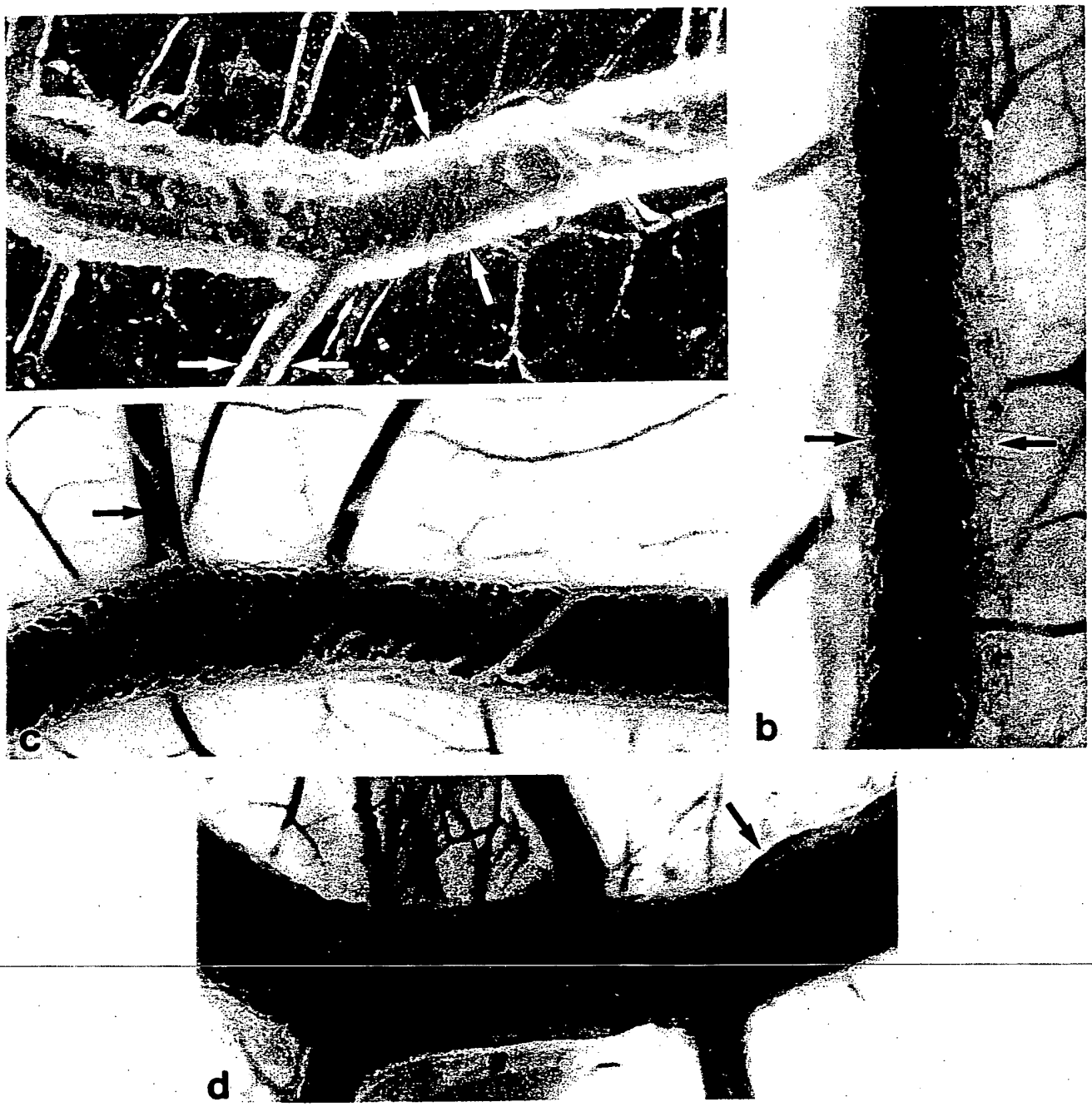
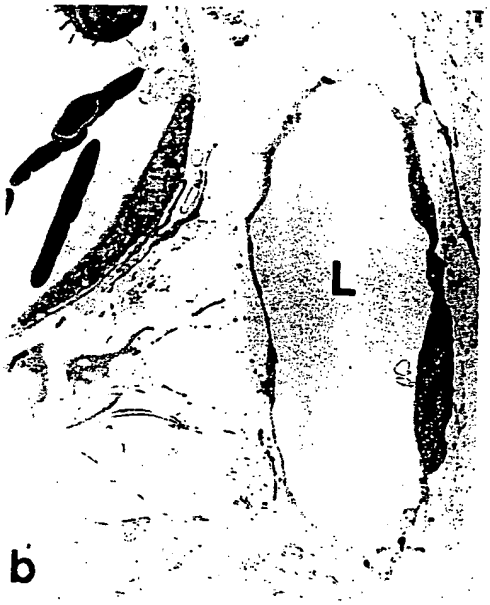
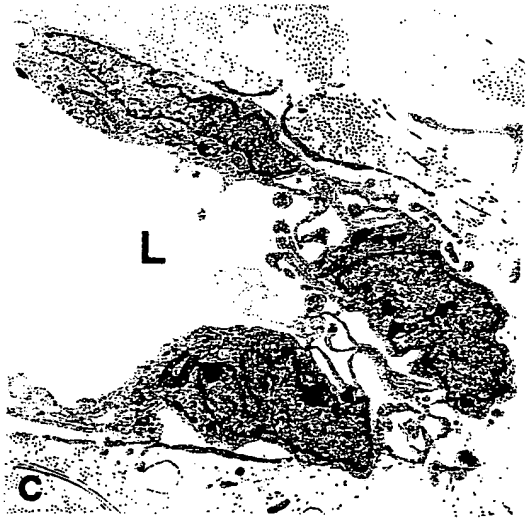


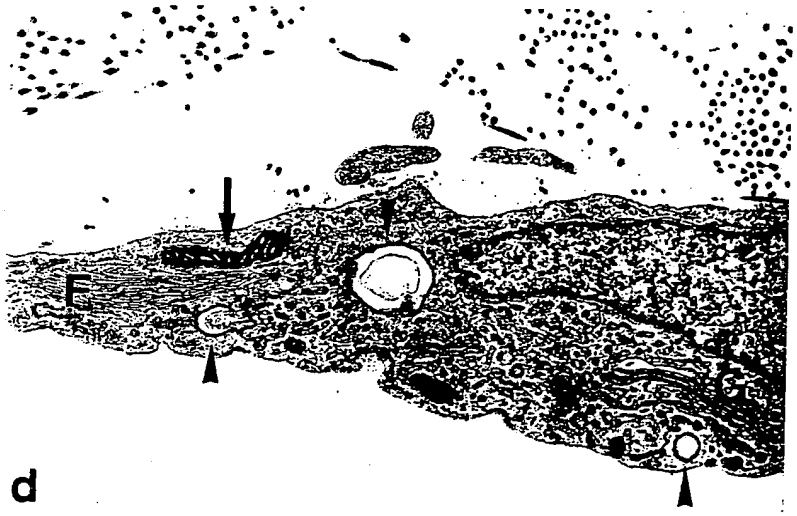
FIG. 1. Intralymphatic injection of Mercox-blue into a 16-day-old chick embryo. (a) A pair of lymphatics (arrows) is present beside arteries and arterioles of CAM. (b) Higher magnification showing an artery accompanied by a pair of lymphatics (arrows). (c) CAM veins accompanied by lymphatics (arrow) and surrounded by a lymphatic plexus. (d) Vein leading to the allantoic stalk, surrounded by a lymphatic plexus. Note formation of lymphatic trunk (arrow). Magnification ×20.

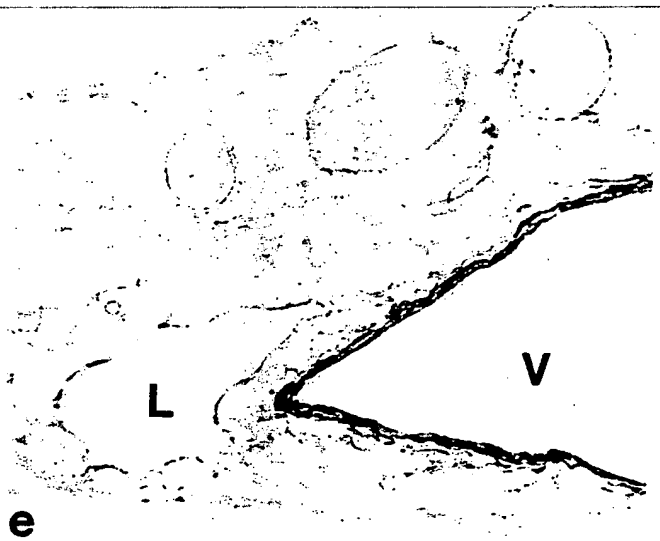
or in semithin sections (Figs. 6e and 6f). However, the heterodimers $VEGF_{121}$/PlGF-1 and $VEGF_{165}$/PlGF-2 were angiogenic (Figs. 5g, 5h, and 6g). An effect of the smallest, non-heparin-binding form could be detected even at some distance from the application area. As before, the lymphatics remained unaffected (Fig. 6g).

The mitogenic effect of $VEGF_{121}$ was studied by monitoring the incorporation of BrdU into the DNA. In previous studies we have found that VEGF induces a three- to four-fold increase of the proliferation rate of endothelial cells of CAM (Wilting *et al.*, 1993; Kurz *et al.*, 1995). We now distinguished between blood vascular and lymphatic endo-









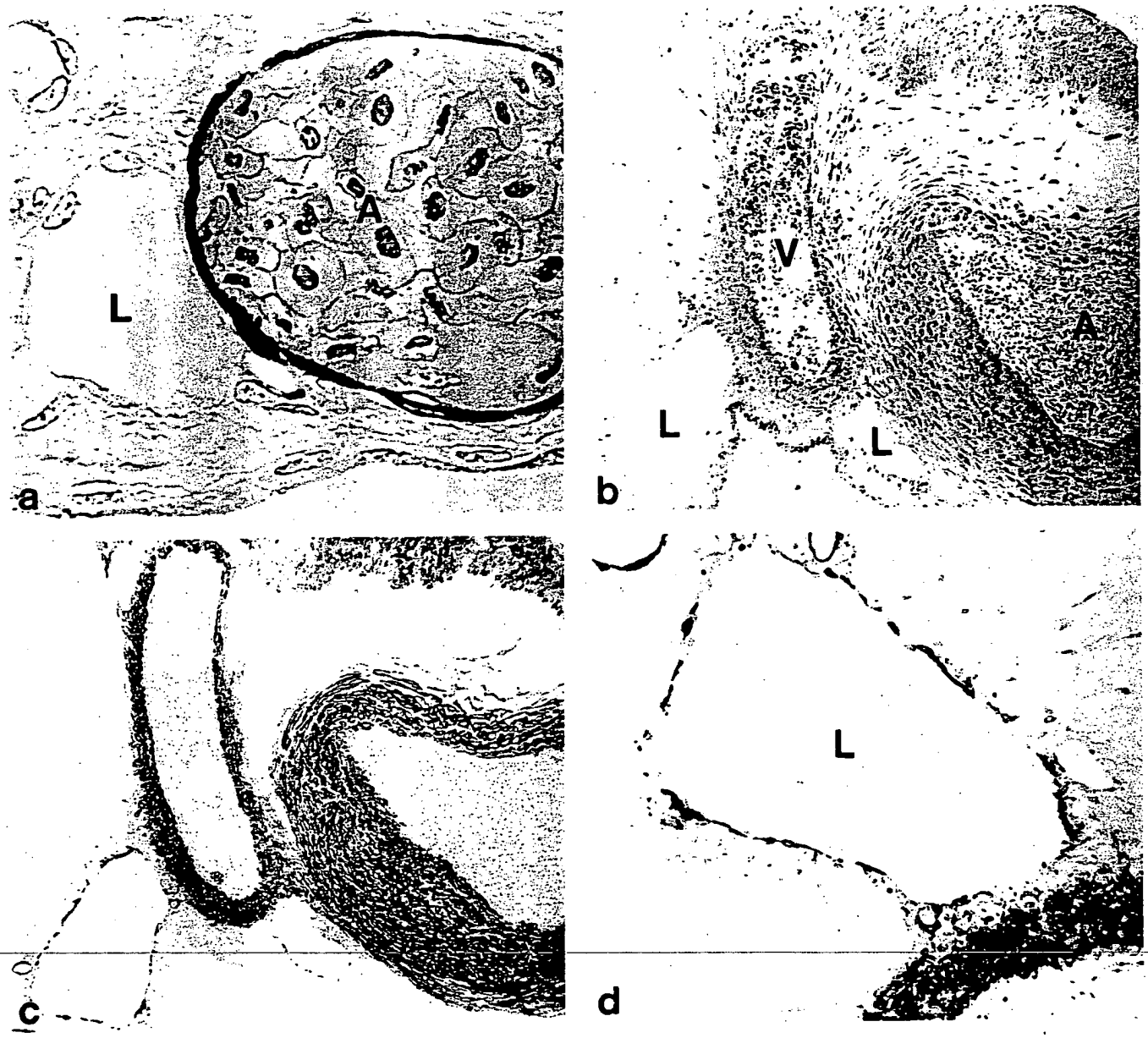


FIG. 3. Semithin sections of CAM and the allantoic stalk of 16-day-old chick embryos. (a, c, d) Anti-α-smooth muscle actin staining. (a) CAM artery (A) invested by smooth muscle cells. The lymphatics (L) do not possess a media. Magnification ×1100. (b) Section showing umbilical artery (A), vein (V), and lymphatic trunks (L). Magnification ×120. (c) Parallel section showing the presence of a media of lymphatic trunks. Magnification ×120. (d) Higher magnification of c. The lymphatic trunk (L) is invested by a single layer of smooth muscle cells. Magnification ×300.

FIG. 2. Structure of CAM lymphatics of 16-day-old chick embryos. (a) Semithin section showing a vein (V) and a lymphatic capillary (L) with its thin endothelial lining. Magnification ×1100. (b) Ultrathin section of a lymphatic capillary. There is no investment by any other cell type. Magnification ×2500. (c) Lymphatic endothelial cells (L) with numerous lamellinodia. Magnification ×3500. (d) Higher magnification of c. Lymphatic endothelial cell with Golgi apparatus (G), microfilaments (F), mitochondria (arrow), and several vesicles (arrowheads). There is no continuous basal lamina. Magnification ×15,500. (e) Semithin section stained for fibronectin. There is weak staining in the stroma and around lymphatic capillaries (L) and strong staining in the media of veins (V). Magnification ×300.

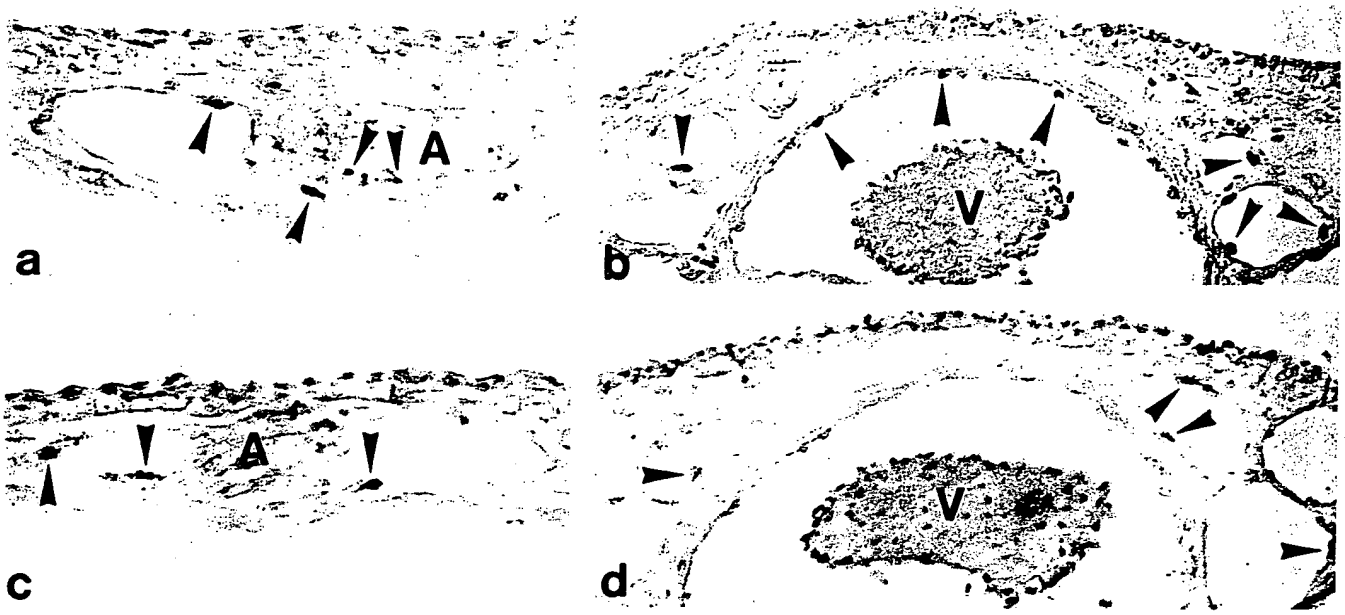
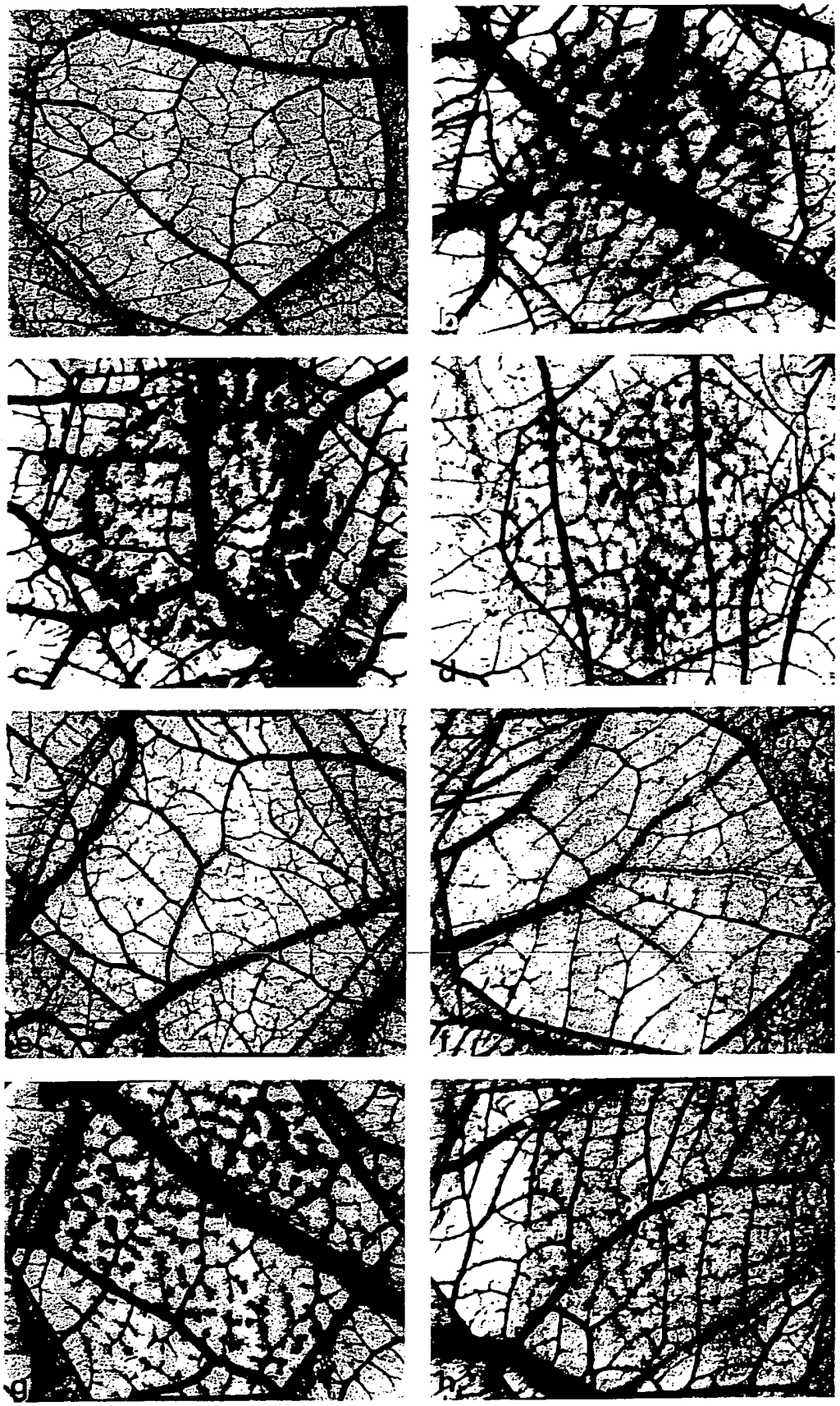


FIG. 4. *In situ* hybridization on paraffin sections of CAM of 13-day-old quail embryos. (a) VEGFR-2/*Quek1* expression (arrowheads) is detectable in endothelial cells of arteries (A) and accompanying lymphatics. Magnification ×600. (b) VEGFR-2/*Quek1* is expressed in endothelial cells (arrowheads) of veins (V) and accompanying lymphatics. Magnification ×400. (c) VEGFR-3/*Quek2* expression (arrowheads) is detectable in endothelial cells of lymphatics. A, artery. Magnification ×600. (d) VEGFR-3/*Quek2* expression (arrowheads) in a lymphatic plexus surrounding a vein (V). Magnification ×400.

thelial cells. A great number of BrdU-labeled blood vascular endothelial cells were found after application of $VEGF_{121}$, whereas those of the lymphatics were negative (Fig. 7).

VEGF-C was produced in insect cells (Kukk *et al.*, 1996; Joukov *et al.*, 1996, 1997) and about 3.3 μg was applied on differentiated CAM. When a 5-μl droplet of VEGF-C-containing solution was dried on the carrier disk, most of the protein was found at the border of the droplet. Therefore, the highest amount of growth factor was arranged in a circle, as has also been demonstrated with other proteins (Wilting *et al.*, 1991). According to the distribution of the growth factor, a circular effect of VEGF-C was visible (Fig. 8a). Within this circle, a weak alteration of the vascular system was observed. However, this was not the main effect. By injecting Mercox-blue into the lymphatics it was possible to demonstrate that in the circular VEGF-C-affected area numerous lymphatics were present (Fig. 8b). High density of lymphatics was observed in the whole application site. A huge lymphatic sinus was located in the circle of highest growth factor concentration. Whereas the normal lymphatics of differentiated CAM were accompanying blood vessels, the newly formed lymphatics could as well be found as isolated vessels. In semithin sections, the lymphatic nature of the VEGF-C induced vessels was also visible. They were huge sinuses with an extremely thin endothelial lining (Fig. 8c). Many folds and endothelial-lined islands were present within the sinuses, indicative of plexus formation. As there were no obvious signs of sprouting, the new lymphatics seemed to grow by intercalation. They were located immediately beneath the chorionic epithelium, where they are never found in normal CAM (Figs. 8c and 8d). In the circular region of highest growth factor concentration newly formed vascular capillaries surrounded the lymphatics (Fig. 8d). The extremely thin endothelial lining of the lymphatics and the absence of a basal lamina were also visible in ultrathin sections (Fig. 8e). Here it was also observed that there was no formation of a media, which could be confirmed in semithin sections stained with an antibody against α-smooth muscle actin (not shown). Proliferation studies revealed a great amount of BrdU-labeled nuclei of lymphatic endothelial cells after 1 and 2 days of VEGF-C application (Fig. 8f), the effect being greater after 1 day.

FIG. 5. Effects of endothelial growth factors applied on differentiated CAM of chick embryos. (a) Control. The carrier does not induce vascular alterations. (b) $VEGF_{121}$ induces angiogenesis. (c) $VEGF_{165}$ induces angiogenesis. (d) $VEGF_{121/165}$ heterodimer induces angiogenesis. (e) PlGF-1 does not induce angiogenesis. (f) PlGF-2 does not induce angiogenesis. (g) $VEGF_{121}$/PlGF-1 heterodimer induces angiogenesis. (h) $VEGF_{165}$/PlGF-2 heterodimer induces angiogenesis. Magnification ×12.



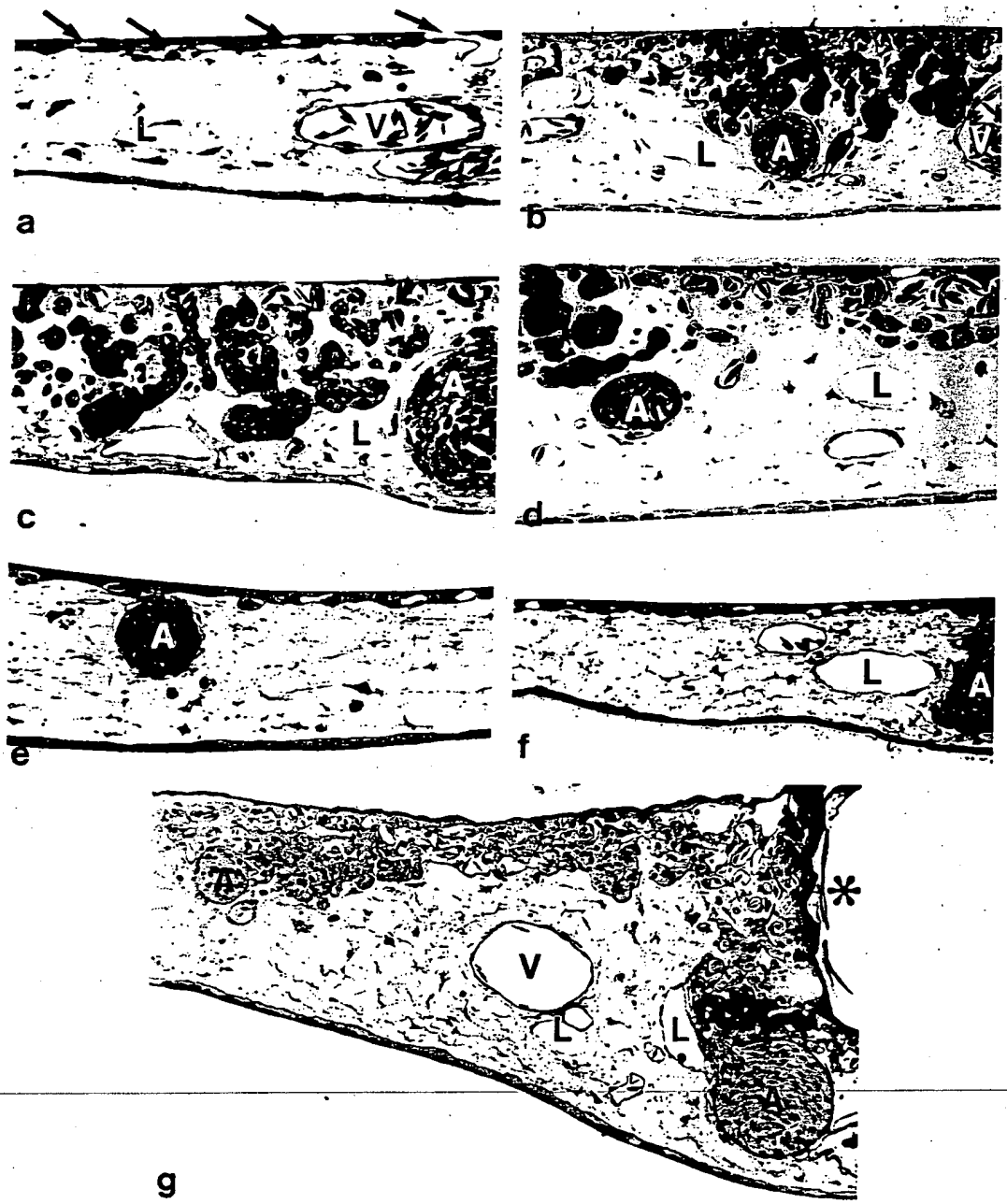


FIG. 6. Semithin sections of growth-factor-treated CAMs corresponding to those in Fig. 5. (a) Control. The capillaries (arrows) are located in the chorionic epithelium. Arteries, veins, and lymphatics are found in the stroma of CAM. Magnification ×450. (b) Effect of $VEGF_{121}$. Numerous capillaries are found in the stroma of CAM. Lymphatics seem to be unaffected. Magnification ×350. (c) Effect of $VEGF_{165}$. Note large amount of blood-filled capillaries. Lymphatics are not affected. Magnification ×450. (d) Effect of $VEGF_{121/165}$ heterodimer. New capillaries are present in the stroma of CAM. The lymphatics are not affected. Magnification ×450. (e) PlGF-1. Neither an angiogenic nor a lymphangiogenic effect is present. Magnification ×450. (f) PlGF-2. There are no signs of angiogenesis or lymphangiogenesis. Magnification ×350. (g) $VEGF_{121}$/PlGF-1 heterodimer. The border of the carrier disk (star) is shown. Due to diffusion, formation of new capillaries in the stroma of CAM is found even at a distance of 0.6 mm from the application site. Lymphatics are not affected. Magnification ×350. A, artery; L, lymphatics; V, vein.

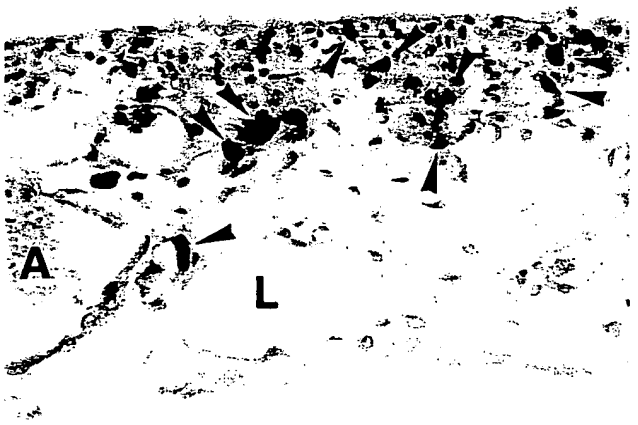


FIG. 7. Proliferation studies with the BrdU/anti-BrdU method after application of $VEGF_{121}$. Numerous BrdU-positive vascular endothelial cells (black nuclei) are present. Lymphatic (L) endothelial cells are negative. A, artery. Original magnification ×400.

DISCUSSION

Lymphatics of CAM Possess Typical Morphology

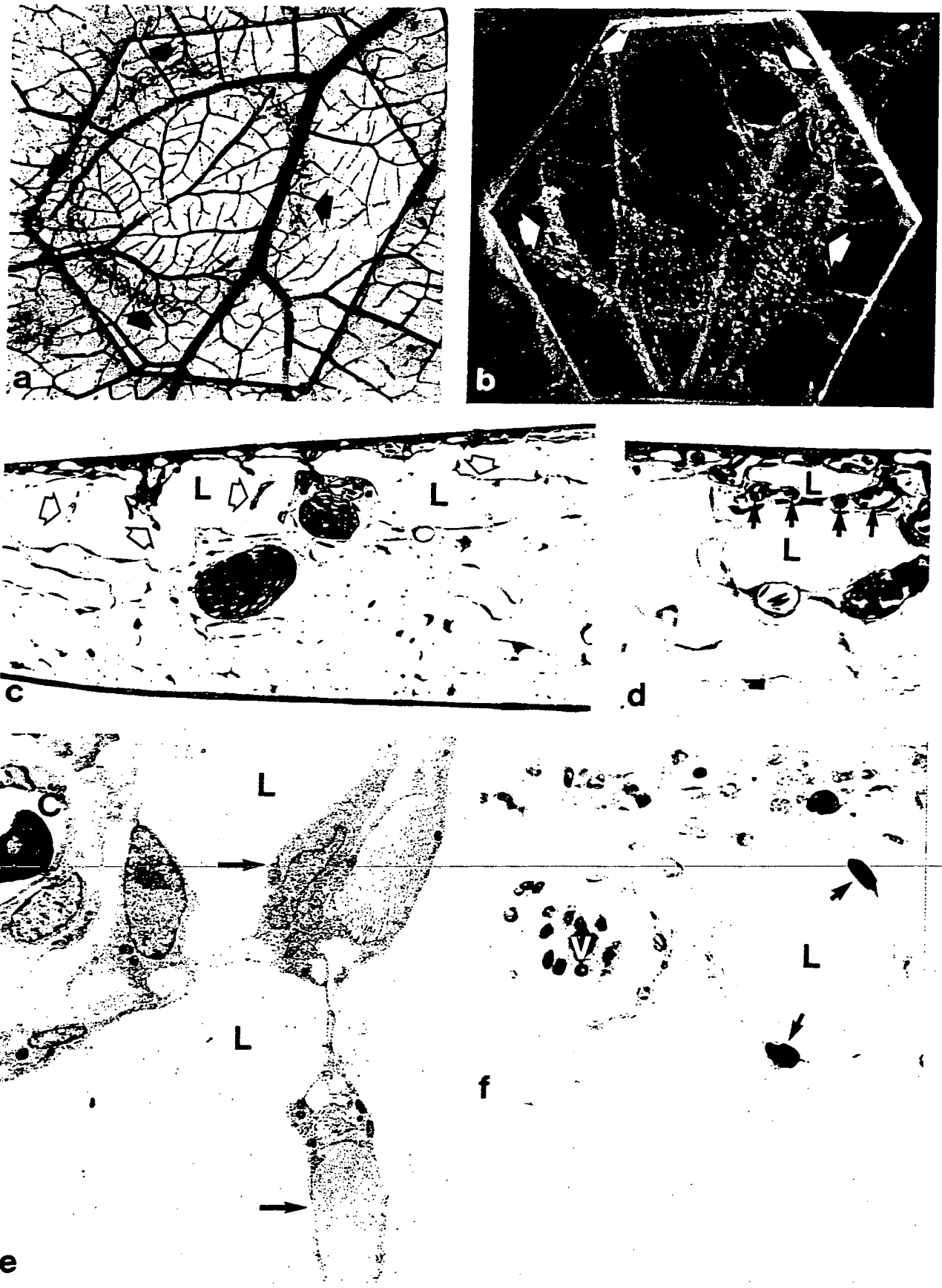
The lymphatics of the chick embryo were first studied by Albrecht Budge during the seventh and eighth decades of the 19th century. After his death, a survey of these studies was published by Wilhelm His (Budge, 1887). As early as 1887, Budge had already noted that the arteries of the CAM are accompanied by a pair of lymphatics which are interconnected. The lymphatics are drained into two lymph-hearts located in the angle between the pelvic and the sacral bones. Furthermore, they are connected to the paired thoracic ducts. These are located adjacent to the aorta and lead to the venous angles. Since the original description of CAM lymphatics by Budge (1887), they have only been briefly mentioned in the textbooks by Hamilton (1965) and Romanoff (1960).

In contrast to lymphatics, the blood vessels of avian CAM have gained considerable attention. In the search for angiogenic factors that may promote tumor vascularization, the CAM assay has been used to assess effects of growth factors. The assay was originally performed on Day 10 of development (Folkman, 1974). In a modified version, differentiated CAM of 13-day-old embryos has been shown to respond selectively to different growth factors (Wilting *et al.*, 1991, 1992). VEGF was observed to induce endothelial cell proliferation, upregulation of VEGFR-2, and angiogenesis, without affecting other cells of CAM (Wilting *et al.*, 1993, 1996; Kurz *et al.*, 1995). Recently, three more proteins sharing approximately 30–40% identity with VEGF have been described and named VEGF-B, VEGF-C, and PlGF (Maglione *et al.*, 1991; Olofsson *et al.*, 1996; Joukov *et al.*, 1996). VEGF-C binds with high affinity to VEGFR-2 (*flk-1*, KDR) and VEGFR-3 (*flt-4*) (Joukov *et al.*, 1996). During development VEGFR-2 is expressed in endothelial cells of blood vessels and lymphatics (Wilting *et al.*, 1997a), and VEGFR-3 becomes restricted to those of lymphatics (Kaipainen *et al.*, 1995; Wilting *et al.*, 1997a). It has therefore been suggested that VEGF-C may exert its activities on lymphatics (Kukk *et al.*, 1996; Wilting *et al.*, 1997b). Lymphatic endothelial cells have been studied *in vitro* (Gumkowski *et al.*, 1987; Witte and Witte, 1987); however, an *in vivo* assay for potential lymphangiogenic factors has not previously been described.

We have studied the lymphatics of avian CAM. Our results support the findings of Budge (1887). A pair of lymphatics is present along all arteries and arterioles of CAM. With increasing size of the arteries, an increasing number of lymphatic capillaries interconnects the pair of lymphatics. Blind-ending extensions are also visible. Veins are also accompanied by lymphatics. Again, the number of lymphatic capillaries and the complexity of the lymphatic plexus increase with increasing size of the veins. Lymph of CAM flows into the embryo. Huge lymphatics are located in the angle between the umbilical vessels. These lymphatics are easily visible with a stereomicroscope. They have to be regarded as lymphatic trunks since they possess a single layer of media smooth muscle cells. In avian CAM, lymphatics do not possess a media and they are made up solely of endothelium.

An extremely thin endothelial lining is one of the characteristics of lymphatic capillaries. Furthermore, their diameter is usually much bigger than that of blood capillaries. They also possess pores, and a basal lamina is missing (Casley-Smith, 1980; Witte and Witte, 1987; Berens von Rautenfeld and Drenckhahn, 1994). Therefore, staining with antibodies against type IV collagen and laminin is negative around lymphatic capillaries and in lymphangiomas (Barsky *et al.*, 1983; Ezaki *et al.*, 1990). The lymphatics of CAM show all characteristics that have been described for normal lymphatic capillaries: huge diameter, porous and very thin endothelial lining, and absence of a basal lamina. From these characteristics, it is possible to recognize lymphatics in semithin sections although, in routine paraffin histology, one cannot be sure to recognize them. However, in CAM, their regular pattern and location beside arteries and veins is a good help for the recognition of lymphatics, even in paraffin sections. Our findings therefore indicate that the regular pattern and the normal structure of CAM lymphatics make this organ highly suitable for the study of lymphangiogenic factors.

Our studies also show that the lymphatics of CAM express VEGFR-2 and VEGFR-3. This expression pattern has also been found in intraembryonic sites during late embryogenesis (Kaipainen *et al.*, 1995; Wilting *et al.*, 1997a). VEGFR-2 is expressed in blood vascular and lymphatic endothelial cells during all stages examined (Wilting *et al.*, 1997a). This receptor has only been studied in context of angiogenesis, and not lymphangiogenesis (Millauer *et al.*, 1993; Shalaby *et al.*, 1995). In early stages of development, VEGFR-3 is expressed by all endothelial cells. Later it becomes restricted to a subpopulation of endothelial cells, disappearing first from the arteries. Expression persists only



in lymphatic endothelial cells, with very few exceptions (Kaipainen *et al.*, 1995; Wilting *et al.*, 1997a). The newly identified growth factor, VEGF-C, binds with high affinity to VEGFR-2 and VEGFR-3 (Joukov *et al.*, 1996; Kukk *et al.*, 1996).

VEGF and VEGF-C Induce Angiogenesis and Lymphangiogenesis, Respectively

The angiogenic potential of VEGF is well established. This growth factor binds to VEGFR-1 (*flt-1*) and VEGFR-2 (Terman *et al.*, 1992; Millauer *et al.*, 1993). Targeted mutations of either the growth factor or its receptors result in early embryonic lethality (Shalaby *et al.*, 1995; Fong *et al.*, 1995; Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). Both $VEGF_{165}$ and $VEGF_{121}$ induce angiogenesis in differentiated CAM (Wilting *et al.*, 1992, 1996). No effect of $VEGF_{121}$ on the lymphatic system was observed in a previous study (Wilting *et al.*, 1996) or in this study. The factor induces expression of VEGFR-2 in endothelial cells, but it does not influence expression of VEGFR-3 (Wilting *et al.*, 1996), to which it does not bind. Here, we have compared the activities of different homo- and heterodimeric forms of VEGF. All forms that contained at least one VEGF chain were capable of inducing angiogenesis. Histologically we have never observed effects in the lymphatics. Since VEGFR-2 is expressed in both types of vessels, differences may reside in the expression of VEGFR-1. It is probable that a combined activation of VEGFR-1 and VEGFR-2 is necessary to induce angiogenesis. However, differential expression of VEGFR-1 in blood vascular and lymphatic endothelium has not been studied to date. VEGF/PlGF heterodimers induce angiogenesis in differentiated CAM. Such heterodimers have been isolated from GS-9L glioma cells (DiSalvo *et al.*, 1995) and have been found in conditioned media of human tumor cell lines (Cao *et al.*, 1996). Therefore, in some tumors this heterodimer may be an important angiogenic factor.

The function of PlGF is much less established. PlGF shares 40% amino acid sequence identity with VEGF, and it binds only to VEGFR-1 (Maglione *et al.*, 1991; Hauser and Weich, 1993; Park *et al.*, 1994). In differentiated CAM, PlGF-1 and PlGF-2 induce neither angiogenesis nor lymphangiogenesis. Activation of VEGFR-1 does not seem to be sufficient to induce angiogenesis. In the avian, however, we have to be aware of the fact that the VEGFR-1 has not been cloned to date. Since PlGF is almost exclusively expressed in the placenta (Park *et al.*, 1994), it may induce placenta-specific characteristics of the vasculature.

Our studies show that VEGF-C is a highly specific lymphangiogenic factor. This is consistent with the expression of its receptors in lymphatic endothelial cells of differentiated tissues (Kaipainen *et al.*, 1995; Wilting *et al.*, 1997a). Whereas VEGFR-2 is expressed in vascular and lymphatic endothelial cells throughout development (Wilting *et al.*, 1997a), expression of VEGFR-3 becomes restricted to those of lymphatics (Kaipainen *et al.*, 1995; Wilting *et al.*, 1997a). The early lymphatics are therefore indistinguishable from blood vessels, which supports the assumption that they are derived from blood vessels (Sabin, 1909; Clark and Clark, 1920). We suggest that the activity of VEGF-C in embryonic tissues is different from that in differentiated tissues. In differentiated CAM, VEGF-C induces a very mild angiogenic response in regions of high growth factor concentration. Preliminary studies in our lab indicate that the angiogenic activity of VEGF is enhanced by VEGF-C. However, the main effect of VEGF-C is induction of new lymphatics. These are located immediately beneath the chorionic epithelium, which indicates that VEGF-C is a chemoattractant for lymphatic endothelial cells. Whereas VEGF induces development of vascular capillaries with relatively small diameter, VEGF-C induces huge lymphatic sinuses, obviously due to proliferation and intercalated growth. By morphological criteria, the lymphatic nature of these vessels can clearly be demonstrated. The great amount of lymphatics in the application area and their unusual location immediately beneath the chorionic epithelium show that they are newly formed.

No growth factors specific for the lymphatic vascular system have yet been described. VEGF regulates vascular permeability and angiogenesis, but it does not promote lymphangiogenesis. Overexpression of VEGF-C, a ligand of VEGFR-3 and VEGFR-2, in the skin of transgenic mice resulted in lymphatic, but not vascular, endothelial proliferation and vessel enlargement (Jeltsch *et al.*, 1997). Thus, VEGF-C appears to induce selective hyperplasia of the lymphatic vasculature, which is involved in the draining and filtering of interstitial fluid, in immune function, in inflammation, and in tumor metastasis. VEGF-C may have a

FIG. 8. Effect of VEGF-C. (a) VEGF-C does not induce gross alteration of the vascular system of CAM. A circular effect (arrows) is only visible in the area of highest growth factor concentration. Original magnification ×12. (b) Intralymphatic injection of Mercox-blue outside the application area. The solution has filled most, but, for technical reasons, not all of the lymphatics in the circular application area. Note the high density of lymphatics in the application area. A huge lymphatic plexus is located in the circle of highest growth factor concentration (arrows). Original magnification ×12. (c, d) Semithin sections of the application area. (c) Newly developed lymphatics (L) located immediately beneath the chorionic epithelium. Note many intraluminally located endothelial cells (arrows), indicative of plexus formation. No other cell types are affected. Original magnification ×450. (d) In the region of highest VEGF-C concentration, some newly formed lymphatics (L) are surrounded by vascular capillaries (arrows). Original magnification ×450. (e) Ultrathin section of the application area. Newly formed lymphatics (L) located adjacent to an intrachorionic capillary (C). Note intraluminally projecting lymphatic endothelial cells (arrows). Original magnification ×3500. (f) Proliferation studies with the BrdU/anti-BrdU method 1 day after application of VEGF-C. Lymphatic (L) endothelial cells (arrows) are BrdU positive. V, Vein. Original magnification ×1000.

role in various disorders involving the lymphatic system and it may prove to be useful for therapeutic lymphangiogenesis.

In summary, our studies show that differentiated CAM comprises a regular pattern of lymphatics. The lymphatic endothelial cells possess ultrastructural characteristics usually described in this cell type. They express VEGFR-2 and VEGFR-3. CAM is therefore a suitable site to study lymphangiogenic factors. We have shown that VEGF homo- and heterodimers induce angiogenesis, but not lymphangiogenesis; that PlGF is neither angiogenic nor lymphangiogenic; and that VEGF-C is the first member of the VEGF family that specifically induces lymphangiogenesis.

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